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EFFECTS OF PETROLEUM OILS ON THE OXYGEN UPTAKE IN RESPIRATION OF PARSNIP AND MUSTARD[1, 2]

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It has been known for several years that certain petroleum fractions are useful as selective herbicides. The effect of these oils on the respiration of species susceptible and resistant to them is, however, not reported to any great extent in the literature. Of the relatively few papers available, the majority are a study of carbon dioxide output (4), and only a few deal with oxygen uptake. Johnson and Hoskins (5) found that a non-herbicidal, neutral, non-peroxidic oil had only a small immediate effect on the O_2 uptake by bean leaf fragments, but toxic oils having high acidic and peroxidic contents resulted in a marked reduction in O_2 uptake immediately after their application. Wedding et al (10), in a study of the long term effects of spray oils on the respiration of citrus leaves, found that the O_2 uptake decreased as a result of oil treatment.

While the effect of petroleum oils on the CO_2 exchange of resistant and susceptible plants has been the main object of study in this laboratory, the desirability of investigating the effects of oil treatment on O_2 exchange was realized. This paper deals with the effects of oils on the respiration of parsnip and mustard leaves or leaf segments as measured in the Warburg respirometer.

MATERIALS AND METHODS

Young plants of parsnip, *Pastinaca sativa* L., and mustard, *Brassica kaber* Wheel. var. Pinnatifida Stokes, were grown in soil in individual pots in the greenhouse as described in an earlier paper. They were then treated as described below and the effect on respiration was measured in a Warburg respirometer. The water in the bath was maintained at 28.1° C and the flasks were moved through 126 oscillations per minute. The physical and chemical characteristics of the oils used in these experiments have been given in table 1 of a previous paper (4).

Entire detached leaves or small segments of leaf tissue were used as experimental material. When entire leaves were to be used, the plants were selected when the 1st 5 or 6 leaves had developed. This required 30 days for mustard and 50 days for parsnip. Usually the 3rd leaf from the base, averaging about 10 cm^2 in area, was detached from the plant and placed in a 15 ml Warburg flask containing 0.2 ml of 10 % KOH in the center well. The respiration of each leaf, as O_2 uptake, was followed for 1 hour in the respirometer to establish a relationship between the respiration of the leaves selected as untreated controls and the test leaves selected for treatment. This relationship was termed "expected rate". The leaves were then removed from the flasks. To saturate the intercellular spaces an eye-dropper was used to treat the test leaves with the oil under investigation. All the leaves were again placed in the flasks for subsequent respiration determinations. The respiration of the untreated control leaves was usually found to remain fairly constant for several hours, although in some experiments it showed a gradual decline in rate with time. In determining the effect of the oil treatment, it was assumed that the expected rate would remain constant throughout the experiment and any variations in this expected rate after oil treatment would be caused by the oil. The results are presented as percent expected rate plotted against time in hours. In each experiment the effect of a similar oil treatment on the appearance of entire plants maintained in the dark was simultaneously observed.

When leaf segments were to be used, the plants were permitted to grow 2 weeks longer until the leaves had reached a larger size. Leaves of similar age and size were then selected from the plants and after removal of the larger veins were cut into 2 mm squares with a razor blade. These were thoroughly mixed and divided into samples of 300 mg fresh weight. Control samples were selected and the remaining samples were treated by immersion in the oil under investigation; the excess oil being allowed to drain. All the samples were then placed in the Warburg flasks with 2 ml of 0.1 M phosphate buffer. Buffers at a pH of 5.6 for mustard and at a pH of 6.6 for parsnip were used to approximate the pH determinations on expressed sap. Two tenths ml of 10 % KOH was placed in the center well for determination of O_2 uptake.

From 2 to 6 experiments were carried out on each phase of the work and the mean results of these experiments are presented. The daily mean respiration

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rates with their standard errors are given for the control plants in the figure legends.

The measurement of CO_2 output in the Warburg was attempted in some experiments, but the method is indirect, being dependent on the measurement of O_2 uptake on a similar sample (8). When leaf segments were used, the samples were paired. One flask was set up with KOH in the center well for O_2 uptake determination, and one without KOH for the determination of the net gas exchange, from which CO_2 output could be calculated. When entire detached leaves were used however, the respiration could not be assumed to be the same for each leaf and it was therefore necessary to measure both O_2 and CO_2 on the

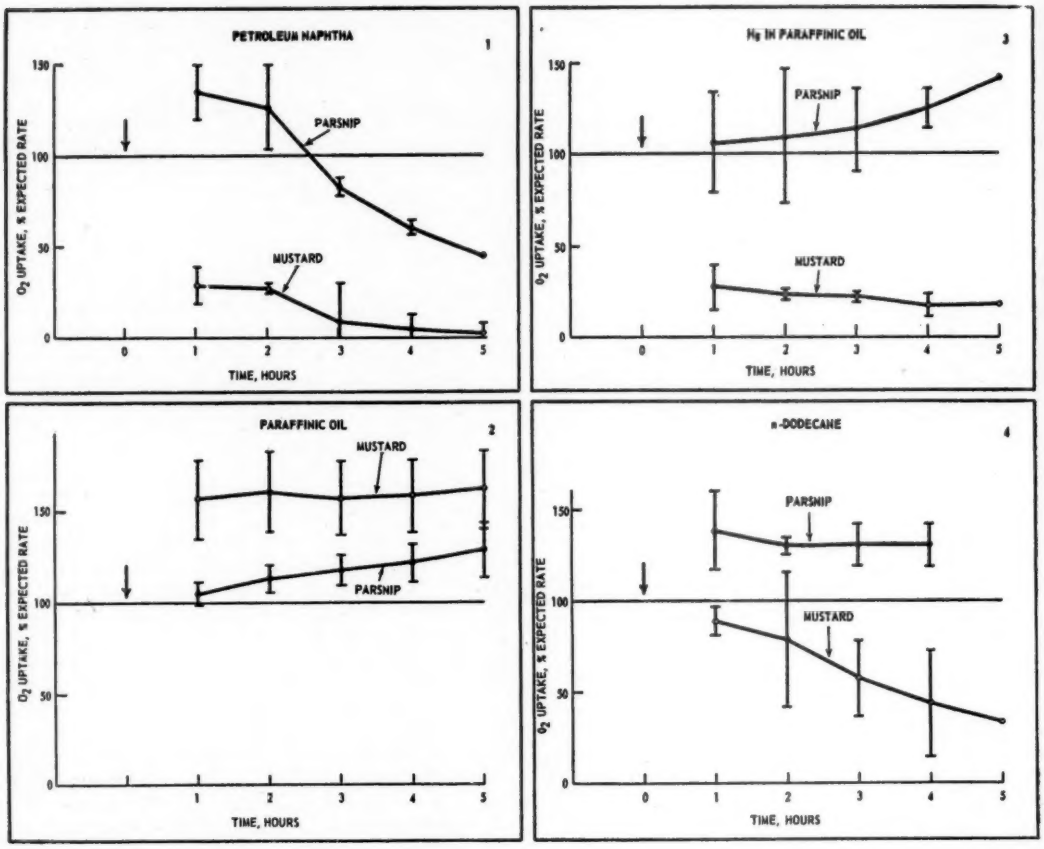

FIG. 1. Effects of petroleum naphtha on the O_2 uptake of entire, detached leaves of parsnip and mustard. Expected rate is the ratio of the mean respiration rates of the control and test leaves before oil was applied to the test leaves. Oil applied at arrow. Vertical lines indicate twice the standard error of the mean of 6 experiments. Daily mean respiration rates and standard error for control leaves: parsnip 9.9 $\pm$ 0.41 μl O_2/hr $\times$ cm^2, mustard 5.7 $\pm$ 0.70 μl O_2/hr $\times$ cm^2.

FIG. 2. Effects of a non-phytotoxic, paraffinic oil on the O_2 uptake of parsnip and mustard leaves. As the results for entire, detached leaves and leaf segments were similar, these results have been combined for calculating the means and standard errors. Oil applied at arrow. Vertical lines indicate twice the standard error of the mean of 8 experiments. Daily mean respiration rates and standard error for control leaves: parsnip 10.1 $\pm$ 1.21 μl O_2/hr $\times$ cm^2, mustard 5.0 $\pm$ 0.64 μl O_2/hr $\times$ cm^2.

FIG. 3. Effects of 300 ppm of Hg in paraffinic oil on the O_2 uptake of entire, detached leaves of parsnip and mustard. Vertical lines indicate twice the standard error of the mean of 2 experiments. Daily mean respiration rates and standard error for control leaves: parsnip 10.2 $\pm$ 0.60 μl O_2/hr $\times$ cm^2, mustard 4.3 $\pm$ 0.66 μl O_2/hr $\times$ cm^2.

FIG. 4. Effects of *n*-dodecane on the O_2 uptake of entire, detached leaves of parsnip and mustard. Oil applied at arrow. Vertical lines indicate twice the standard error of the mean of 2 experiments. Daily mean respiration rates and standard error for control leaves: parsnip 10.2 $\pm$ 0.87 μl O_2/hr $\times$ cm^2, mustard 5.2 $\pm$ 0.79 μl O_2/hr $\times$ cm^2.

same leaf. This was done in a few experiments by alternately removing and replacing the KOH in the center well, but this method is at best only an approximation of the CO_2 evolved.

Experimental Results

Effects of Petroleum Naphtha: Petroleum naphtha, boiling range 149 to 204° C is a selective herbicide widely used as a spray for weeding carrots and parsnips (6). The effects of this oil on the respiration of entire leaves of the resistant parsnip plant and the susceptible mustard plant are given in figure 1. The data represent the mean results for O_2 uptake of a number of experiments and are expressed as percent expected rate for entire leaves. Following application of petroleum naphtha to entire parsnip leaves the O_2 uptake increased in the 1st hour and then gradually declined to about 50 % of the expected rate by 5 hours after treatment. When entire mustard leaves were treated with petroleum naphtha, the O_2 uptake dropped rapidly to 30 % of the expected rate in the 1st hour.

The effect of petroleum naphtha on the O_2 uptake of leaf segments was similar to that of the entire leaves. The results of CO_2 measurement on oil-treated leaf segments indicated that the CO_2 output of treated parsnip and mustard leaf segments followed closely the O_2 uptake. Parsnip plants were generally undamaged by this oil, but mustard plants were killed.

Effects of Paraffinic Oil: The effects of a non-phytotoxic paraffinic oil, boiling range 204 to 260° C on the respiration of entire, detached leaves of parsnip and mustard are given in figure 2. This oil increased the respiration of the leaf tissue in both species. It had no apparent effect on the appearance of either parsnip or mustard plants after the oil had evaporated. For entire parsnip leaves, the increase in O_2 uptake was gradual, reaching 130 % of the expected rate by 5 hours after oil treatment. The effect of the oil on the O_2 uptake of entire mustard leaves was not consistent from one experiment to another although there was an average increase to 160 % of the expected rate. Similar results were obtained for the O_2 uptake of leaf segments. The CO_2 output of mustard leaf segments only was measured and was found to be equivalent to the O_2 uptake.

Effects of Mercury Added to the Paraffinic Oil: Hg in the form of the oil-soluble methyl-9-acetoxymercuri-10-methoxystearate as prepared by Ralston et al (7) when added to the paraffinic oil was previously found to give selective herbicidal properties (4). The effects of 300 ppm of Hg in the paraffinic oil on the O_2 uptake of entire, detached parsnip and mustard leaves are given in figure 3. The effect on parsnip leaves resembled that of the paraffinic oil alone (fig 2), but on mustard it resembled that of the petroleum naphtha (fig 1). Parsnip plants treated with this oil remained normal in appearance when the oil evaporated, but mustard plants wilted and eventually died as a result of the oil treatment.

Effects of Pure Hydrocarbons: The paraffin, *n*-dodecane and the aromatic, *p*-cymene were chosen as being representative of the paraffins and aromatics contained in a petroleum naphtha. The effects of *n*-dodecane on the O_2 uptake of entire, detached leaves of parsnip and mustard are given in figure 4. The O_2 uptake of parsnip increased to 130 % of expected rate in the 1st hour after treatment where it remained for 4 or more hours, while the O_2 uptake of mustard gradually decreased to 30 % by 5 hours after treatment. The *n*-dodecane had a selective action, causing no visible injury to parsnip plants after the oil had evaporated but completely killing mustard plants.

The *p*-cymene caused the O_2 uptake of both parsnip and mustard to cease by 2 hours after treatment (not shown) and both parsnip and mustard plants were killed by this aromatic hydrocarbon.

Discussion

The rate of O_2 uptake of parsnip and mustard leaf tissue was found to be decreased by herbicidal oils and to be increased by non-herbicidal oils. Although petroleum naphtha caused the rate of O_2 uptake of resistant parsnip leaf tissue to increase after treatment (fig 1), the subsequent drop in O_2 uptake was an indication of damage to the tissue. This damage was likely a result of the oil remaining in close proximity to the tissue in the closed system of the respirometer. Under this condition, in agreement with the view of Crafts (2) that resistance to the oil is a matter of degree rather than a peculiar characteristic of the resistant species, the level of tolerance of the parsnip tissue to the oil was exceeded, and the tissue was damaged by the oil.

The ability of parsnip to tolerate an oil to a greater extent than mustard was not likely, under the conditions of the experiments, to be a result of anatomical or morphological differences which in the field would affect the dispersal of the oil. Dallyn (3) and van Overbeek and Blondeau (9) suggest that resistance to oil treatment is a result of inherent differences in the cell cytoplasmic membrane, which permits a differential penetration of petroleum hydrocarbons. The results of the experiments in which Hg was added to the paraffinic oil would indicate that some such protective measure was operative in parsnip (fig 3). The respiration of parsnip was increased by this highly toxic element while that of mustard was greatly reduced.

The results with pure hydrocarbons indicate that the paraffin fraction as well as the aromatic fraction of petroleum naphtha has a toxic effect on susceptible species. Mustard showed little resistance to either the paraffin *n*-dodecane or the aromatic *p*-cymene. Parsnip was highly resistant to *n*-dodecane, but was not resistant to *p*-cymene.

The effects of oil treatment on the respiration of parsnip and mustard tissues may be compared with the effects of dinitrophenol (DNP) on the respiration of various plant tissues, Beevers (1). Treatment with a non-herbicidal oil such as the paraffinic oil

resulted in an increase in the respiration rate similar to that caused by dilute concentrations of DNP. Treatment with a herbicidal oil such as petroleum naphtha resulted in a decrease in the respiration rate similar to that caused by stronger concentrations of DNP.

Summary

A Warburg respirometer was used to study the effects of petroleum oils on the respiration of parsnip and mustard leaves. A non-herbicidal, paraffinic oil, boiling range 204 to 260° C, increased the respiration of both parsnip and mustard with no visible injury to the plants. The herbicidal oils, petroleum naphtha, boiling range 149 to 204° C, a pure paraffin, *n*-dodecane and 300 ppm of Hg in the paraffinic oil all caused the respiration of the resistant parsnip to increase with no visible injury to the plant and the respiration of the susceptible mustard to rapidly decrease with the death of the plant. The pure aromatic, *p*-cymene, caused the respiration of both parsnip and mustard to cease by 2 hours after treatment and both plants were killed.

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COMPARATIVE RESPONSES OF CARBON DIOXIDE OUTBURST AND UPTAKE IN TOBACCO [1, 2]

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Evolution of CO_2 from leaves of tobacco and several other terrestrial species is very rapid during the first several seconds of darkness immediately following a period of illumination (2, 4, 5), as is shown for coffee in figure 1. van der Veen (8) reported results that have been interpreted by others as indicating a similar phenomenon. The similarity of records is, however, superficial and misleading. Although both sets of records have CO_2 concentration on the ordinal axis and time on the abscissal axis, results are not directly and simply comparable.

In the 1st method a leaf was in a recirculating system, and rate of increase or decrease of concentration in the system (slope of a recorder tracing) was taken as a direct measure of rate of CO_2 evolution or uptake by the leaf. In van der Veen's method the leaf chamber was part of an open-end system (non-recirculating), and the CO_2 concentration of air flowing from the chamber (height of a tracing) yielded a direct measure of rate of CO_2 evolution or uptake. Thus, in this 2nd method, slope of a given tracing is not a measure of photosynthetic rate but is instead a measure of rate of change of photosynthetic rate. Presumably, a clearly defined postilluminational outburst would make the following kind of record on van der Veen's apparatus: An initial horizontal tracing at a height well below the input concentration reference mark (as the illuminated leaf takes up CO_2 at a nearly constant rate, making outflow concentration well below input) followed by a rapid swerve upward to a peak well above the input reference mark (as uptake stops and the leaf begins to evolve CO_2 rapidly, now making outflow concentration greater than input) followed by a slower swerve downward to a new horizontal slightly above input reference (as CO_2 evolution decelerates gradually to normal dark level, but with outflow still greater than input). No such record was presented.

Gaffron and Rosenberg (6) have reported postilluminational outbursts from the alga, Scenedesmus. Other similar phenomena have reviewed recently by Kok and Spruit (7).

Conceivably, outburst of CO_2 might result from a

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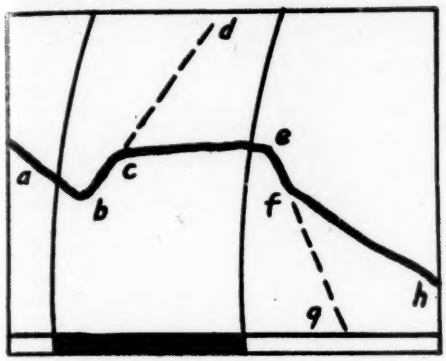
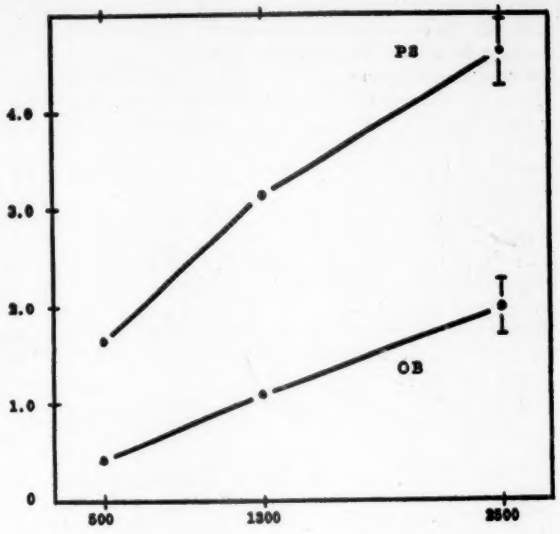
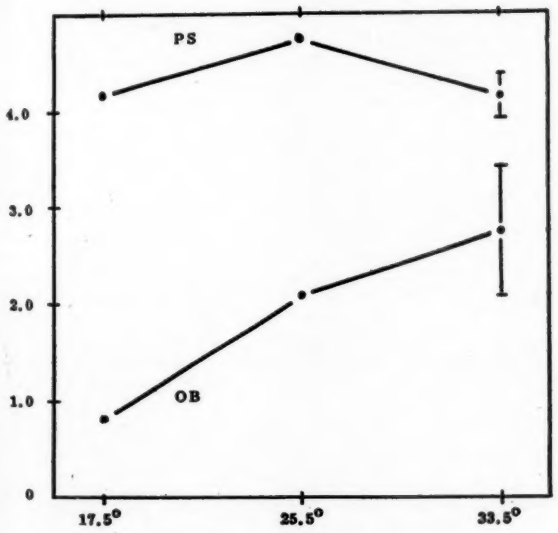
[2] Experimental work was done at Brooklyn Botanic Garden.







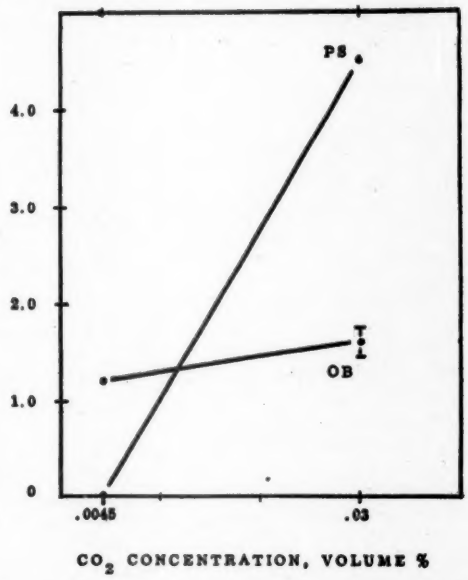


FIG. 1. (*upper left*). Part of a recorder chart showing time-course of CO_2 concentration in a closed system containing an intact coffee leaf. Slope of segment ab is rate of CO_2 uptake by illuminated leaf. Light was turned off for 90 seconds (black bar) beginning at time marker on left. Slope of bcd is initial rate of CO_2 evolution in darkness (outburst). Slope of ce is subsequent dark respiration rate. This chart is from Decker and Tió (5), and is included here only to illustrate the outburst phenomenon graphically. A recorder was not needed or used for the work reported in the present paper.

FIG. 2 (*upper right*). Effect of light intensity on apparent photosynthesis (PS) and postilluminational outburst (OB) of tobacco leaves at 25.5° C and 300 ppm CO_2. Ordinal unit is μg CO_2 per second per dm² of leaf area. Each point is mean of 70 observations. Confidence ranges are 5 % tse (t × standard error of mean) and were computed from residual error terms of analyses of variance.

FIG. 3 (*lower left*). Effect of temperature on apparent photosynthesis and outburst of tobacco at 2500 ft-c and 300 ppm. Each point is mean of 35 observations. Otherwise similar to figure 2.

FIG. 4 (*lower right*). Effect of CO_2 concentration on apparent photosynthesis and outburst of tobacco at 2500 ft-c and 25.5° C. Each point is mean of 25 observations. Otherwise similar to figure 2.

slight delay between deceleration of photosynthesis and deceleration of "combustion", a hypothetical process postulated by Burk and Warburg (1) to accompany photosynthesis as a closely linked back-reaction. If outburst does result from such a lag, it too should appear closely linked to photosynthesis, and the 2 should respond similarly to changes of experimental conditions. The present study was undertaken to test this possibility.

The plants (hybrid tobacco), apparatus and general methods have been described (3). A leaf, still attached to a plant, was sealed in an illuminated chamber that was coupled in closed series with an air pump and an infrared gas analyzer. An increase of CO_2 in the system was considered a measure of CO_2 evolution by the leaf; a decrease was considered a measure of CO_2 uptake or apparent photosynthesis.

The routine operating procedure was as follows. A leaf was left for about 20 minutes at 2500 ft-c and 25° to 26° C before measurements were begun. An excess of CO_2 was added. The resulting surging in the system quieted in a few seconds and CO_2 concentration began to fall smoothly and rapidly. The decrease was timed with a stopwatch over a short range that represented uptake of 39.76 μg of CO_2 by the leaf and that had its midpoint at 300 ppm. This was done by watching the indicator move across 2 fixed points on the dial of a microammeter attached to the amplifier of the gas analyzer. An opaque cover was then put on the leaf chamber. After about 8 seconds, concentration began to rise rapidly. The rapid rise was timed immediately over another short range representing production of 8.6 μg of CO_2. The opaque cover was removed, CO_2 was added, and the procedure was repeated. Rates of uptake and evolution were computed by dividing 39.76 μg and 8.6 μg by appropriate observed time in seconds. Leaf area was computed from the weight of a piece of aluminum foil cut to the pattern of the leaf.

Five measurements each of apparent photosynthesis (CO_2 uptake) and of CO_2 outburst (evolution) were made at 2500 ft-c, at 1300 ft-c and at 500 ft-c with each of 14 tobacco plants. Mean rates computed from these 420 observations show that both processes increased with light intensity (fig 2).

With 7 plants randomly selected from the 14, light intensity was returned to 2500 ft-c immediately after the last measurement at 500 ft-c. Five measurements of apparent photosynthesis and of outburst were made on each plant at 17.5° and then at 33.5° C. Corresponding observations for 25.5° C were taken from the preceding experiment. Mean rates computed from these 210 observations show that apparent photosynthesis was reduced slightly at both 17.5° and 33.5° C; whereas outburst was increased at 33.5° and markedly reduced at 17.5° C (fig 3).

With 5 of the 7 plants, quintuplicate measurements were made at 25.5° C of apparent photosynthesis at 300 ppm; of outburst at 300 ppm immediately following each observation of photosynthesis; of the CO_2 compensation point (that concentration at which uptake becomes zero); and of outburst immediately following photosynthesis at the compensation point. Means computed from these 100 measurements show that outburst was reduced only slightly at the low concentration; whereas apparent photosynthesis was, of course, reduced to zero (fig 4).

Because photosynthesis and outburst diverged in their responses to temperature and to CO_2 concentration, they are not inseparably linked phenomena. Hypothetical "combustion" was postulated by Burk and Warburg (1) as being cyclically bound to photosynthesis. Therefore, the outburst observed here does not lend support to their hypothesis.

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SOME EFFECTS OF TEMPERATURE AND CARBON DIOXIDE CONCENTRATION ON PHOTOSYNTHESIS OF MIMULUS[1]

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It has been proposed that photosynthetic responses to an altitudinal gradient of CO_2 might influence altitudinal distribution of some plants (1). The present work was undertaken as a preliminary test of this hypothesis and yielded no support to it. Additionally, the study yielded information about temperature effects on carbon dioxide compensation concentration, apparent photosynthesis at simulated high altitude, apparent respiration, and respiration during photosynthesis.

For simplicity of expression, the following definitions will be adhered to throughout this paper: photosynthesis—total amount of CO_2 reduced, regardless of origin; apparent photosynthesis—measurable decrease of CO_2 in a closed system containing an illuminated green leaf; respiration—amount of CO_2 produced endogenously and either reused or evolved; apparent respiration—measurable increase of CO_2 in a closed system containing an illuminated leaf.

MATERIALS AND METHODS

Mimulus was selected for this study because altitudinal distribution of races and species has been studied intensively (5) and because abundant clonal material was readily available. Two clones were used. One (clone 6546–5) was an individual of *M. cardinalis* Dougl. originally from an elevation of about 150 feet near the Pacific coast at Los Trancos Creek, San Mateo County, California. The other (clone 6546–3) was an F_1 hybrid between the Los Trancos form of *M. cardinalis* and a subalpine plant of *M. lewisii* Pursh. from 10,700 feet elevation in the Sierra Nevada Mountains at Slate Creek Valley, Mono County, California. According to Nobs (6) the Los Trancos strain grew well at low altitude but did not survive when transplanted to 4,500 feet altitude; whereas the hybrid flourished at low altitude, at 4,500 feet and at about 10,000 feet.

All propagules were from cuttings taken in May 1957, started in sand, and grown in soil in 4-inch pots in a greenhouse at Stanford, California. Experiments were done during July 1957. Six pairs of opposite leaves on 5 plants of the parent clone and 6 pairs on 3 plants of the hybrid clone were used.

The apparatus used was a modification of one already described (2). It consisted of a leaf chamber, a small air pump, and an infrared gas analyzer (Beckman L/B 15 coupled to a Varian recording potentiometer) in closed series. The analyzer recorded continuously the concentration of CO_2 in the system. A change of concentration was considered a direct measure of uptake or evolution of CO_2 by the plant material enclosed in the leaf chamber.

The routine procedure was as follows. An intact branch tip, still attached to an intact potted plant and bearing 1 pair of opposite leaves, was sealed in the chamber and left at a standard illumination of 2000 ft-c (300 watt, internal-reflector, tungsten filament flood lamp) and a constant temperature for 15 to 30 minutes before measurements were begun. Then CO_2 was added to raise the concentration to about 500 ppm. The subsequent uniformly decelerated decrease of CO_2 in the system was recorded. When concentration had fallen below 100 ppm, more CO_2 was added and a duplicate tracing was made (fig 1). This time, concentration was allowed to fall to compensation, that is, to that low value at which there was no net gain or loss of CO_2 by the leaf. Then a scrubber bottle of aqueous NaOH was put in the system, and CO_2 was reduced to less than 50 % of compensation concentration. The scrubber was removed, and the subsequent increase of concentration (apparent respiration) was recorded. This procedure was duplicated. The chamber was then darkened and duplicate tracings of CO_2 evolution were made at 300 ppm. The whole procedure was repeated at 2 other temperatures before the leaves were removed for area measurement. Leaf area was computed from the weight of a piece of aluminum foil cut to the pattern of the leaf.

Each of the 6 possible sequences of 3 temperatures (20°, 30° and 40° C) was used once with each clone. This design was intended to eliminate any subtle but consistent extraneous effect (e.g., sequence, length of time under the lights, exposure to low CO_2 concentration, etc.) from comparisons between temperatures or between clones by distributing it uniformly over temperatures and clones. Preliminary tests revealed no massive effect of the experimental procedure that needed to be taken into further account.

RESULTS

Rates of CO_2 uptake and evolution were computed from slopes of lines drawn tangent to recorder tracings as shown in figure 1 (example: at 300 ppm, 1 mv = 2.34 μg CO_2 in the 239 ml system; $2.34 \times 21.5 / 60/0.23\ dm^2 = 3.65\ \mu g/sec/dm^2$ for the tracing shown). Duplicated rates of apparent photosynthesis at 300, 200 and 100 ppm, of apparent respiration at varied low concentrations, of dark respiration at 300 ppm, and a single observation of the CO_2 compensation concentration were thus obtained for each pair of leaves at each temperature (except that there was usually no apparent photosynthesis at 100 ppm and 40°). Mean rates and concentrations computed from these data are shown in figure 2.

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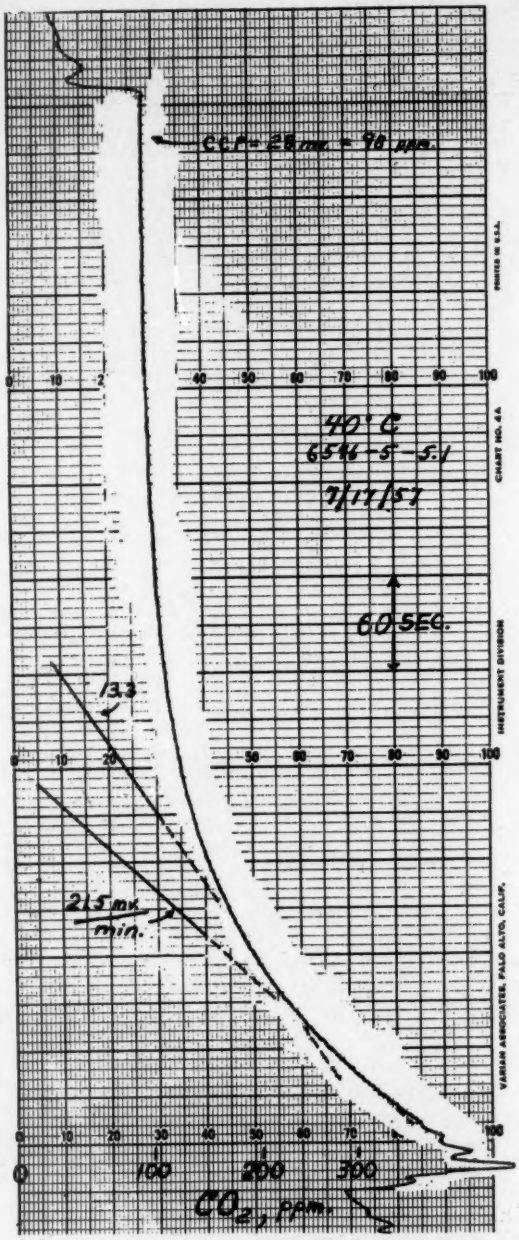

FIG. 1. Photograph of a recorder tracing showing the decrease of CO_2 concentration in a closed system containing a pair of intact Mimulus leaves illuminated at 2000 ft-c. Some grid marks were washed over with white ink to increase contrast. Recorder pen moved upward. Straight lines are drawn tangent to the tracing at 200 and 300 ppm. CCP is CO_2 compensation concentration.

TABLE I

AVERAGE RESPIRATION RATES* FOR 6 SETS OF MIMULUS LEAVES IN DARKNESS; AND RATIO OF APPARENT PHOTOSYNTHESIS OF SAME LEAVES AT 2000 FT-C AND 300 PPM TO RESPIRATION (P/R)

	R			P/R		
	20°	*30°*	*40°*	*20°*	*30°*	*40°*
Parent clone	0.15	0.29	0.46	21.8	9.8	4.7
Hybrid clone	0.22	0.39	0.63	12.3	7.2	2.9

*In μg CO_2/sec/dm^2.

The confidence interval (fig 2) was computed from the error term of an analysis of variance (table II). It can be interpreted as indicating that if the experiment were rerun with similar conditions and materials, mean values should be expected to deviate from those shown by more than this amount only about once in 20 trials.

Table II is based on only the 144 measurements of apparent photosynthesis represented in figure 1 by the 12 points for 20° and 30° C at 100, 200 and 300 ppm. Because there were few measurements at 100 ppm and 40° C, all measurements at 40° were excluded to simplify computations. Measurements of apparent respiration were excluded because they were made at a variety of concentrations and were thus subject to additional variance. Because compensation concentration was subject to an entirely different kind of variance (resulting in horizontal rather than vertical dispersion as seen in figure 2), a separate analysis of variance was made for these measurements (table III).

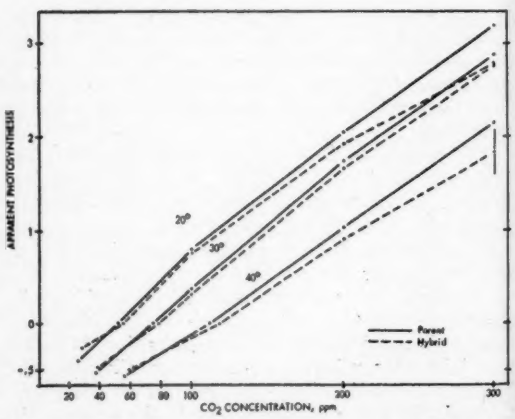

FIG. 2. Effects of air temperature and of CO_2 concentration on apparent photosynthesis of intact leaves of Mimulus. Illumination was 2000 ft–c. Ordinal unit: μg CO_2 per second per dm^2 of leaf area. Points at zero apparent photosynthesis represent means of 6 observations each, all others are means of 12.

TABLE II

ANALYSIS OF VARIANCE OF DATA REPRESENTED IN FIGURE 1 BY UPPER 12 VALUES AT 20° AND 30° C

SOURCE OF VARIATION	DEGREES OF FREEDOM	VARIANCE
Clones (Cl)	1	0.355
Individuals (I)	10	1.989
Subtotal	11	
Temperatures (T)	1	1.532
T-Cl	1	0.058
T-I	10	0.224
Subtotal	23	
Concentrations (Co)	2	33.028
Co-Cl	2	0.076
Co-T	2	0.112
Co-T-Cl	2	0.041
Error (Co-I + Co-T-I)	40	0.172
Total	71	

DISCUSSION

SIMILARITY OF RESPONSES OF THE TWO CLONES: Inspection of figure 2 suggests only slight differences between the clones. This similarity is confirmed more rigorously by table II. Marked and consistent differences in the responses of the 2 clones to temperature or concentration would have resulted in much larger interaction variances (T-Cl and Co-Cl). A differential response to concentration that varied with temperature would have resulted in a larger 3-way interaction (Co-T-Cl).

This work does not establish that the 2 clones are necessarily similar in their photosynthetic responses to altitude changes. It simply establishes that no consistent difference was revealed. There are several possibilities that need investigation before the original hypothesis can be ruled out for even these 2 clones: 1) Perhaps differences in photosynthetic mechanism develop only when plants are actually grown at high altitude. 2) Perhaps reduced partial pressure of CO_2 does not simulate high altitude realistically. 3) Variance between individuals of the same clone was surprisingly high and perhaps masked otherwise significant differences.

TEMPERATURE DEPENDENCE OF CO_2 COMPENSATION CONCENTRATION: The increase of compensation concentration with temperature shown in figure 2 is consistent with that reported by Egle and Schenk (4) and by Thomas and Hill (8). It is at variance with results of earlier studies reviewed by Rabinowitch (7.) Thomas and Hill ascribe this dependence to the fact that the temperature coefficient for respiration is probably much larger than that for photosynthesis, and no alteration of their interpretation is proposed here.

TABLE III

ANALYSIS OF VARIANCE OF DATA REPRESENTED IN FIGURE 2 BY THE 6 VALUES FOR COMPENSATION CONCENTRATION (APPARENT PHOTOSYNTHESIS = 0)

SOURCE OF VARIATION	DEGREES OF FREEDOM	VARIANCE
Clones	1	165
Individuals	10	84
Subtotal	11	
Temperatures	2	11,643
T-Cl	2	12
T-Cl-I	20	107
Total	35	

SUGGESTED EXPLANATION FOR COMPENSATION CONCENTRATION: The well-known fact, here demonstrated again, that compensation concentration exceeds zero probably indicates that a photosynthesizing leaf does not reuse directly all endogenous CO_2, even when photosynthesis is CO_2-limited. At this low concentration, light-saturated photosynthesis is clearly CO_2-limited. If all endogenous CO_2 were reused directly, a leaf should continue to take up external CO_2 in proportion to its availability (because ability to consume CO_2 photosynthetically exceeds ability to produce CO_2 in respiration), and the curve of apparent photosynthesis vs CO_2 concentration would pass through the origin. The fact that the curve actually passes to the right of the origin indicates that some endogenous CO_2 diffuses into the external system before it can be reused; and at compensation, uptake of external CO_2 is diminished until it just equals this leakage.

INCREASED IMPORTANCE OF HIGH TEMPERATURE AT SIMULATED HIGH ALTITUDE: Although proportional concentration of atmospheric CO_2 remains nearly constant at 300 ppm irrespective of altitude, actual concentration varies directly with total atmospheric pressure and thus decreases with altitude. Actual concentration at 15,000 feet altitude is about one half that at sea level. Presumably, apparent photosynthesis at 15,000 feet and 300 ppm would be the same as at sea level and 150 ppm. As shown in figure 2, an increase of temperature from 20° to 40° C decreased average apparent photosynthesis of parent plants about 33 % at 300 ppm and about 73 % at 150 ppm. This suggests that limitation of apparent photosynthesis (and therefore of dry weight increment) by high temperature may become more important at high altitude.

EFFECTS OF TEMPERATURE AND CO_2 CONCENTRATION ON APPARENT RESPIRATION: Mean values for apparent respiration are shown as negative values for apparent photosynthesis in figure 2. These fit nearly linear extensions of the lower ends of the curves for apparent photosynthesis and thus tend to validate linear extrapolations made in an earlier study (2). Apparent respiration increased with temperature and with decreased CO_2 concentration.

EFFECT OF TEMPERATURE ON RESPIRATION DURING PHOTOSYNTHESIS: In an earlier study (2) respiration during photosynthesis was estimated from a curve of apparent photosynthesis vs CO_2 concentration by inserting a hypothetical curve for photosynthesis vs CO_2 concentration and measuring vertical distance between the 2 curves. This method is nearly identical

with one that is perhaps more familiar—that of extrapolating an empirical curve back to zero CO_2. It is more complete logically in that it makes explicit the assumptions that are implicit in the latter method: the curve for photosynthesis vs CO_2 originates at the origin, is linear at the lower end, and is parallel to the corresponding curve for apparent photosynthesis. Although these assumptions are reasonable, they are untested and are not required in the following and 3rd method.

For simplicity, discussion is limited to 2 curve segments of figure 2: parent plants at 20° C, from 100 to 300 ppm; parent plants at 40° C, from compensation to 300 ppm. These segments are nearly parallel, that is, temperature effect on apparent photosynthesis was independent of CO_2 concentration.

Apparent photosynthesis is the resultant of photosynthesis minus respiration, and any effect on apparent photosynthetic rate is explainable in terms of rate changes of photosynthesis and respiration. Photosynthesis is CO_2-dependent, and thus a temperature effect on photosynthesis would vary with CO_2 concentration. If such an effect were involved in the response of apparent photosynthesis to temperature, the segments would diverge at high CO_2 concentration. They do not, and this leaves 1 other obvious possibility: respiration.

Respiration of a photosynthetic leaf in darkness is essentially independent of CO_2 concentration over the 100 to 300 ppm range (3). Presumably, respiration in the light is likewise independent. Therefore, the CO_2-independent effect of temperature on apparent photosynthesis most likely resulted entirely from a temperature effect on respiration; and vertical distance between the 2 curve segments yields a measure of the difference of respiration rates during photosynthesis at 20° and at 40° C (1.04 μg/sec/dm^2).

Appropriateness of this interpretation for the present data depends on whether the apparent parallelism is real or fortuitous. Although the analysis of variance reveals no divergence, this cannot be established with certainty because of the large experimental error. However, the fact that the corresponding segments for hybrid plants are also nearly parallel is confirmatory.

Comparative Effect of Temperature on Respiration in Light and Darkness: As indicated in the preceding section, average respiration rate of parent plants during photosynthesis probably increased 1.04 μg/sec/dm^2 as temperature increased 20°. Average respiration rate of the same leaves in darkness increased 0.31 μg/sec/dm^2 with the same temperature increase (table I). Respiratory acceleration was thus about 3.3 times greater in light than in darkness. Presumably, rates were proportional to accelerations, and thus respiration rate was about 3.3 times greater in light than in darkness.

Summary

Effects of 3 temperatures (20°, 30°, 40° C) on apparent photosynthesis of 2 clones of Mimulus were studied over a wide range of CO_2 concentrations. Compensation concentration increased with temperature. High temperature inhibition of apparent photosynthesis was markedly greater at simulated high altitude than at sea level. Apparent respiration increased with temperature. Respiration during photosynthesis was evidently about 3.3 times greater than respiration in darkness.

The experimental plants were grown by Dr. W. M. Hiesey. He, Dr. C. Stacy French and Mr. Harold Milner made many helpful suggestions. Experimental measurements were made mostly by Mrs. Ruth Elliot.

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EFFECT OF ACID SCARIFICATION ON LUPINE SEED IMPERMEABILITY[1]

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Varying proportions of hard seed occur among strains of blue lupine (*Lupinus angustifolius*). These hard seed may be rendered permeable by scarification with acid. Since the basic action of this treatment in altering permeability has not been investigated, this study was designed to determine the effect of acid scarification on the path of liquid entry into blue lupine seed. Research on other legumes has shown that liquids enter the seed by different pathways in different species (1, 6, 7, 9).

In this study a hard-seeded strain of sweet blue lupine was used. When seeds of the above strain were soaked in dye for periods ranging up to 2 weeks, 50 to 75 % of the seeds were found to be permeable. They absorbed dye through the seed coat as a whole, the entry not being limited to any particular region. In the seeds which remained in the dye solution for 2 weeks without swelling (termed hard seed), the cuticle was stained and in 17 % of these seeds some dye had penetrated to the tracheid bar underlying the hilum region (fig 1).

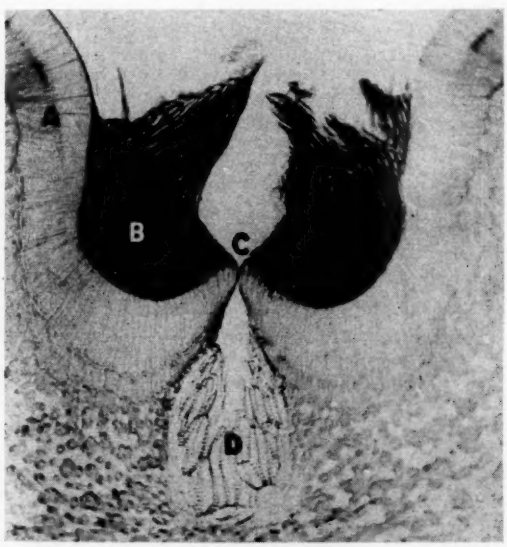

FIG. 1. Cross section of the hilum region of lupine seed. A, palisade epidermis; B, counter palisades; C, hilum fissure; D, trachied bar.

[1] Received July 16, 1958.

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The fact that the dye penetrated the cuticle but did not penetrate the seed coat of the "hard" seeds agrees with the conclusion of most workers that the impermeable layer of the testa is at the outer edge of the palisade epidermis. This is the location of the "light line," an optical phenomena much discussed in the literature (1, 3, 6, 8). The entry through the strophiole reported for other legume seeds (2, 7) did not occur in this strain.

To study the effect of sulfuric acid treatment on the liquid entry, hard seeds were selected by soaking the seeds in water for a week or more and saving the unswollen seeds for study. These seeds were placed in concentrated sulfuric acid for 3 hours. This treatment removed the cuticle of all the seeds and eroded pits through the testa of a small percentage of them.

Seeds treated as above were placed in blue dye and removed at intervals for observation. In all cases in which the seeds were removed before the entire seed was stained, dye entry had occurred through the hilar fissure into the tracheid bar or through pits eroded through the testa.

Observations of cross sections of the hilum region after scarification revealed that the overlying parenchyma was severely hydrolyzed and that the counter palisades were partially hydrolyzed. The palisade epidermis showed hydrolysis only where pits were formed.

Hyde (4) found that the counter palisades and the palisades of the hilum region control moisture uptake from the air. When the internal moisture of the seed exceeds the external moisture the hilum opens, permitting drying; with reverse conditions the hilum closes, preventing moisture uptake.

To determine the extent to which the hydrolysis of the counter palisades region negated this process, both scarified and unscarified seeds were placed in chambers in which atmospheric moisture was controlled with either a moist blotter or calcium chloride. Observations with a wide field microscope revealed that in the sound seed the hilar fissure was open in the dry chamber and closed in the moist chamber. In the scarified seeds the hilar fissure was open under both conditions.

Since the hilar fissure is merely closed by pressure (fig 1) it was thought that a surfactant might be effective in permitting penetration of this region. Neither this nor a fat solvent was effective in rendering the seed permeable.

To determine the effect of swelling and redrying on the permeability of the hard seeds, the testas were pierced with a needle and the seeds were permitted to swell in water. They were then dried rapidly at

43° C and the hole was sealed with beeswax. When placed in the dye solution 90 % of these seeds took up liquid through the strophiole. In the remaining 10 % the liquid entered through the seed coat generally. In no case was it primarily through the hilum. Examination of these seeds after the preliminary soaking revealed cracks in the strophiole of most of the seeds. This observation is in accord with that of Kuhn (5). Of the thousands of seeds observed, only 1 seed not treated as above had liquid entry through the strophiole.

Summary

Examination of the path of liquid entry into impermeable seeds of *Lupinus angustifolius* (a hard-seeded sweet blue strain) after they were scarified in sulfuric acid indicates that entry occurs through either the hilar fissure or pits eroded through the testa. The impermeable layer of the testa is located at the outer edge of the palisade epidermis. Entry was permitted through the hilar fissure due to partial hydrolysis of the counter palisades which normally cause the hilum to close when the moisture outside the seed exceeds that on the inside.

The only time that the strophiole is important in uptake of liquid is after the seeds have taken up water and been redried. This process causes a cleft to be formed in the strophiole.

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SOME FACTORS WHICH AFFECT THE SYNTHESIS OF CHLOROGENIC ACID IN DISKS OF POTATO TUBER[1, 2]

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The work of Neish and his colleagues (9, 10) on the synthesis of aromatic compounds in buckwheat indicates that shikimic acid is a precursor to phenolic constituents such as caffeic acid. Chlorogenic acid, a phenol present in a wide variety of plants, is an ester of caffeic acid and quinic acid, the latter being quite similar to shikimic acid in structure. Unfortunately little is known of the mechanism of biosynthesis of this interesting phenol. The recent observations of Johnson and Schaal (3, 4) and of Kúc, et al (6) have shown that, in contrast to the outer periderm, the pulp of the potato tuber is almost devoid of chlorogenic acid. Kúc (5) has further shown that disks of pulp tissue, nevertheless, are capable of producing this compound. Consequently, tissue from the inner core of the potato tuber appears to offer a relatively simple system for study of the pathways by which chlorogenic acid is synthesized.

The purpose of the work reported in this paper, then, is to investigate the effects of environmental factors on the synthesis of chlorogenic acid. Data are also given which offer an explanation for the difference in distribution of this substance between periderm and pulp.

Materials and Methods

Chlorogenic acid synthesis was studied in disks, 12.5 mm in diameter and 1 mm thick, sliced with a hand microtome from cylindrical plugs cut out of Kennebec potato tubers. The tubers were kept at 7° C until used. Disks were sliced from the inner part of the tuber only, the outer 5 to 8 mm of tissue and skin being discarded. Each experimental treatment consisted of a sample of 10 disks (weighing about 1.5 g) which were withdrawn at random from the washed slices, blotted lightly, and placed in 5 ml of the appropriate solution contained in a 9 cm Petri dish. The covered dish was held at room temperature (23 to 25° C), in the dark, until the disks were ready for assay. All culture solutions contained 25 ppm of Neomycin sulfate to control bacterial contamination. This addition had no effect on synthesis of chlorogenic acid. The data are presented as the total chlorogenic acid content of each sample. Treatments were run in

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[2] This work was supported in part by a grant from the National Science Foundation.

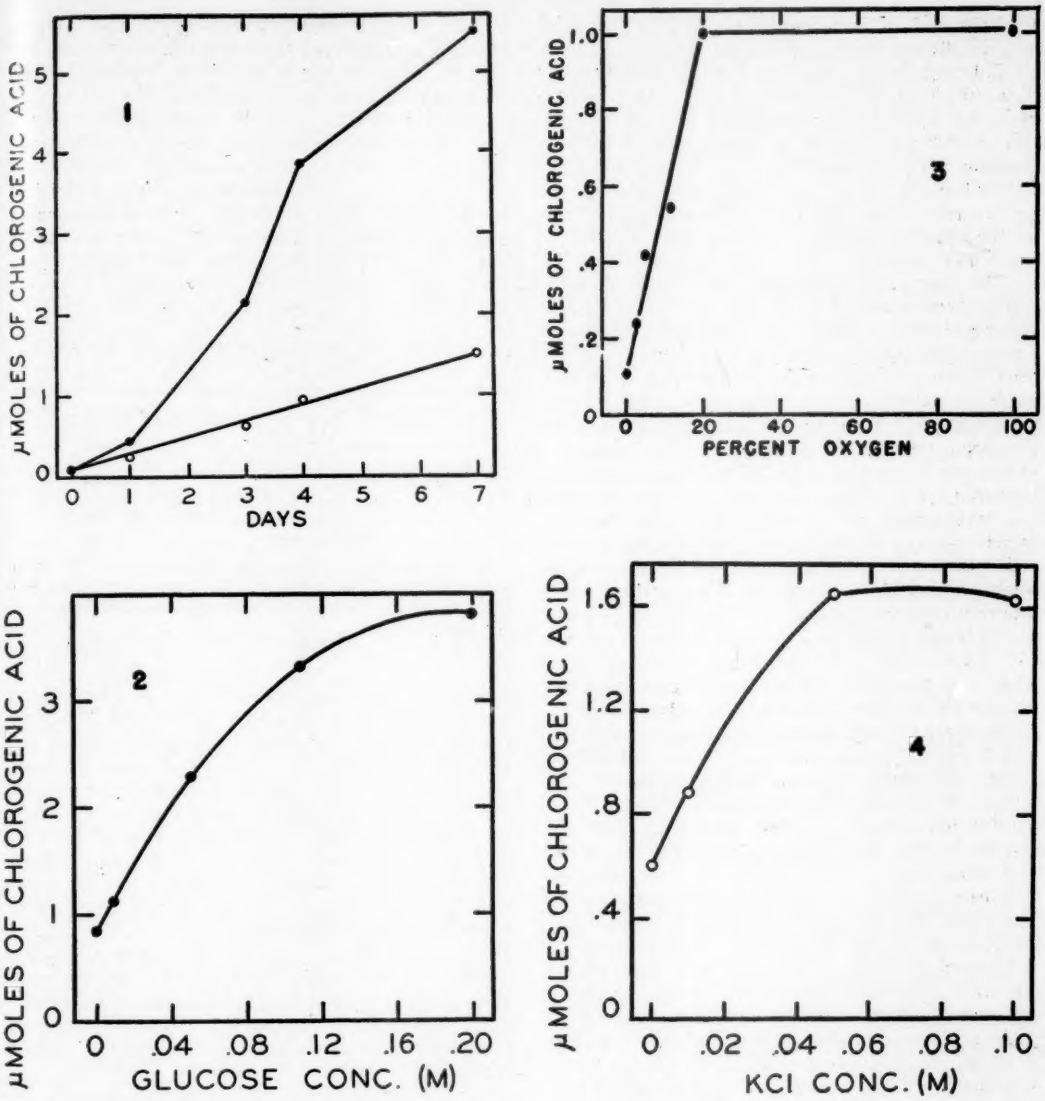

FIG. 1. Time course of synthesis of chlorogenic acid in potato tuber discs. ●, culture solution 0.1 M glucose and 25 ppm of Neomycin sulfate. ○, distilled water containing 25 ppm of Neomycin sulfate.

FIG. 2. The effect of different concentrations of glucose on synthesis of chlorogenic acid in potato tuber disks. Determinations made at end of 72 hours. All culture solutions contained 25 ppm of Neomycin sulfate.

FIG. 3. Effect of oxygen pressure on synthesis of chlorogenic acid. Disks on moist filter paper in sealed flasks were assayed after 72 hours of culture. Each flask was flushed daily with the appropriate mixture of air and oxygen or nitrogen.

FIG. 4. The effect of different KCl concentrations on synthesis of chlorogenic acid in potato tuber disks at the end of 72 hours. All culture solutions also contained 25 ppm of Neomycin sulfate.

duplicate, and the difference between duplicate samples was usually less than 10 % of the mean.

Chlorogenic acid was extracted by grinding the disks in 15 ml of 95 % ethanol with a Ten Broeck glass homogenizer. The suspension in a 25 ml volumetric flask was brought to volume with the ethanol washings of the homogenizer and filtered. Either 2.5 or 5 ml of the clear, filtered extract were placed on an alumina column and assayed for chlorogenic acid by the modified Hoepfner nitrous acid reaction as previously described (11).

The absorption spectrum of the initial alcoholic filtrate from cultured disks was similar to that of chlorogenic acid, indictating that this substance is a major ultra-violet absorbing component of the extract. Ascending paper chromatograms of evaporated alcoholic extracts verified this observation. These chromatograms were run on Whatman no. 1 paper with acetic acid : HCl : water (6 : 1 : 3), 1-butanol : acetic acid : water (6 : 2 : 2), the organic phase of 1-butanol : 2 *N* HCl (1 : 1), and the aqueous phase of *n*-butyl acetate : acetic acid : water (5 : 10 : 75) as solvents. In all solvents, the most intensely fluorescent band had an R_f the same as that of authentic chlorogenic acid. As many as 5 fluorescent bands appeared with the butanol : HCl and butanol : acetic acid solvents. All gave a positive Folin phenol test (1). As anticipated, the eluate of the band the R_f of which was the same as that of chlorogenic acid also had the absorption spectrum of this substance. When chromatograms containing all 5 bands were sprayed with the Hoepfner reagents (2, 7), the band believed to be chlorogenic acid turned bright yellow while several of the other bands became orange. All of these bands turned red with alkali. To determine how much of the material reactive in the chlorogenic acid assay was actually located in the chlorogenic acid band, a butanol : HCl chromatogram was cut into strips, and the eluates of the strips were assayed for chlorogenic acid. About 80 % of the reactive material came from the chlorogenic acid band. Consequently, a correction factor based on this figure was used to calculate the number of micromoles of chlorogenic acid present. Interestingly enough, all treatments which gave rise to a synthesis of chlorogenic acid also appeared to stimulate the production of the other phenolic substances.

Results

In confirmation of previous work (3, 6), disks removed from the inner tissue of the potato tuber contained almost no chlorogenic acid, i.e., less than 0.2 micromoles per gram of fresh weight. However, as shown in figure 1, they began to synthesize chlorogenic acid when immersed in a shallow layer of water, and maintained a steady rate of synthesis for at least 7 days. It can also be seen that glucose markedly affected the synthesis of chlorogenic acid stimulating the rate 3- to 4-fold. The optimal concentration of glucose for synthesis was approximately 0.1 M (fig 2). Sucrose had a similar stimulatory effect.

The low concentration of chlorogenic acid in freshly cut disks suggested that some factor necessary for synthesis was limiting or was absent within the tuber. The cold temperature of storage (7° C) was not responsible for the lack of synthesis since tuber taken from storage and placed at room temperature showed no increase in chlorogenic acid concentration.

In order to test the possibility that a diminished availability of oxygen to the tissue prevented synthesis, disks were placed in different depths of culture solution. Table I shows that limiting the oxygen supply by such treatment had a marked effect on the synthesis of chlorogenic acid. Completely submerged disks synthesized no chlorogenic acid whatsoever. Even the thin film of solution covering the disks under standard conditions (5 ml of solution) decreased the amount of chlorogenic acid formed when comparisons were made with disks completely exposed to the atmosphere. The lack of oxygen in the totally submerged disks was evidenced by the fact that these disks remained white, whereas the surface of disks from the other treatments turned light brown.

Table I

Effect of the Depth of the Culture Fluid on Synthesis of Chlorogenic Acid

Depth of culture fluid	μM chlorogenic acid
Initial content	0.15
8 to 10 mm (disks completely submerged)	0.14
0.8 to 1 mm (disks covered by a thin film of water; standard condition)	0.63
0 (disks not submerged)	1.14

Completely submerged disks were bathed in 50 ml of distilled water (containing 25 ppm Neomycin). Disks not submerged were placed on filter paper moistened with 2 ml of solution.

Samples were assayed after 72 hours of culture.

The importance of an adequate supply of oxygen for the synthesis of chlorogenic acid was demonstrated directly by varying the partial pressure of oxygen to which the disks were exposed. Figure 3 indicates that maximal synthesis was not obtained until a concentration of 20 % oxygen was reached.

Further evidence that lack of oxygen limited synthesis in tuber tissue was provided by an experiment in which the thickness of disks was changed. Table II

Table II

Effect of Disk Thickness on Synthesis of Chlorogenic Acid

Thickness of disks mm	μM chlorogenic acid
0.5	0.92
1.0	0.73
2.0	0.63

Samples of 20 disks 0.5 mm thick, 10 disks 1.0 mm thick, and 5 disks 2.0 mm thick were assayed after 72 hours of culture in distilled water containing Neomycin.

shows that increasing the thickness from 0.5 mm to 2 mm decreased the amount of chlorogenic acid synthesized per unit weight by one third. This result indicates that diffusion of oxygen through 2 mm of tissue is sufficiently hindered to curtail chlorogenic acid synthesis. The amount of chlorogenic acid produced by the standard 1 mm disks was intermediate between that of the two extremes.

In these experiments equal weights of tissue were assayed. That is, 5 disks each 2 mm thick were used in the one treatment while 20 disks 0.5 mm thick were used in the other. Consequently, the area of wound surface present in one sample was 4 times that of the other. Since no such corresponding increase in synthesis of chlorogenic acid occurred, wound reactions or "wound respiration" is apparently not involved.

Potassium chloride is known to stimulate utilization of sugar and respiration in potato disks (8). Figure 4 shows that KCl also increases production of chlorogenic acid. A maximal effect was obtained with 0.05 M KCl. At plasmolytic concentrations above 0.1 M, the tissue became brown and the chlorogenic acid content decreased just as in disks treated with high concentrations of glucose. The maximal KCl stimulation, although varying from experiment to experiment, was always considerably less than the corresponding stimulation of chlorogenic acid synthesis by glucose. Addition of KCl to an optimal concentration of glucose (0.1 M and above) produced no further stimulation of synthesis, but rather inhibited the production of chlorogenic acid (table III). Only

TABLE III

EFFECT OF GLUCOSE AND KCl ON SYNTHESIS OF CHLOROGENIC ACID

GLUCOSE CONC (M)	μM CHLOROGENIC ACID −KCl	μM CHLOROGENIC ACID +KCl*
0	0.86	2.03
1×10^{-2}	1.12	2.24
5×10^{-2}	2.29	2.40
1×10^{-1}	3.33	3.14
2×10^{-1}	3.82	2.16

All samples were assayed after 72 hours of culture. The initial chlorogenic acid content was .16 μM per sample (see fig 2 also).

* The KCl concentration was 0.05 M.

when glucose concentrations were sub-optimal did KCl enhance the glucose effect.

The salt effect was not limited to KCl alone (see table IV). The lack of stimulation by potassium phosphate was unexpected but reproducible, and no explanation can be offered at present. The small effect of NH_4Cl is in part attributable to the fact that disks in this salt, although they remained turgid, were noticeably darker brown than those exposed to any other treatment. This suggests that some of the chlorogenic acid synthesized had been oxidized to the brown pigments observed.

TABLE IV

EFFECT OF SALTS ON SYNTHESIS OF CHLOROGENIC ACID

SALT*	μM CHLOROGENIC ACID
Water	0.70
Potassium chloride	1.83
Potassium nitrate	1.82
Potassium phosphate buffer, pH 6.8	0.70
Calcium chloride	1.49
Ammonium chloride	1.04

All samples were assayed after 72 hours of culture. The initial chlorogenic acid content was 0.15 μM per sample.

* Each salt was present at a concentration of 0.05 M.

Because of the inter-relationships mentioned above (see Introduction) between shikimic acid and quinic and caffeic acids, these compounds were tested for their ability to stimulate synthesis of chlorogenic acid. Table V shows that at half the concentration, quinic acid was as effective as glucose, while shikimic acid produced only a slight stimulation. Caffeic acid proved to be much too toxic to test in this manner.

TABLE V

EFFECT OF QUINIC AND SHIKIMIC ACIDS ON SYNTHESIS OF CHLOROGENIC ACID

CULTURE SOLUTION	μM CHLOROGENIC ACID
Water	0.74
0.1 M glucose	2.02
0.05 M potassium quinate, pH 4.5	1.98
0.05 M " shikimate, "	0.98

Samples were assayed after 72 hours of culture. The initial chlorogenic acid content was 0.16 μM per sample.

DISCUSSION

The present data indicate that synthesis of chlorogenic acid requires a relatively high oxygen tension. The sensitivity to the supply of oxygen may explain why the inner tissue contains little chlorogenic acid even though this phenol can be produced from endogenous substrates. Indeed, the availability of oxygen can account for the biochemical differentiation of the potato tuber, at least in respect to the production of chlorogenic acid. The slow rate of diffusion of oxygen through the skin into the potato establishes an environmental gradient which limits synthesis to the outer layer of tissue.

Both glucose and quinic acid stimulate the synthesis of chlorogenic acid markedly, whereas shikimic acid appears to have no effect other than a salt stimulation. If glucose and quinic acid were only acting indirectly by stimulating general metabolic processes, then shikimic acid, which resembles quinic acid closely in structure, might be expected to produce similar

effects. Since this has not been observed, the data suggest that glucose and quinic acid exert their effects by giving rise to precursors of chlorogenic acid.

Summary

Chlorogenic acid formation has been shown to occur in disks of potato tuber tissue which are initially almost devoid of this compound. Glucose, quinic acid, and, to a lesser extent, salts such as KCl stimulate the synthesis of chlorogenic acid. Availability of oxygen is an important factor in the synthesis of the acid since, under reduced oxygen concentrations, little or no formation of chlorogenic acid could be detected. The sensitivity to oxygen may well explain the presence of chlorogenic acid in the outer few mm of the potato tuber in contrast to its absence in the pulp.

The authors wish to express their appreciation to Dr. H. B. Vickery for his helpful discussion and criticism of this paper.

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EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID AND 2,4-DINITROPHENOL ON THE UPTAKE AND METABOLISM OF EXOGENOUS SUBSTRATES BY CORN ROOTS [1, 2, 3]

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There are many similarities between 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-dinitrophenol (DNP) in their action on plant metabolism. Both of these compounds may inhibit salt accumulation (10, 12), increase respiration (1, 6, 8, 13), inhibit growth (3, 6) and uncouple oxidative phosphorylation in mitochondrial preparations (5, 14). Whether these similarities are superficial or whether they result from a similar mode of action of the two compounds is still an open question (4).

This paper reports the results of an investigation on the effect of DNP on the uptake and metabolism of exogenous substrates by root tips from 2,4-D-treated and buffer-treated corn seedlings. It is concluded that, although both 2,4-D and DNP promote catabolism, they differ in their effect on uptake of the substrates by the root tips.

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Materials and Methods

Corn seedlings (*Zea mays* L., var. Funks G-50) approximately 60-hours-old were used as plant material. The seedlings were placed with their roots in 10^{-2} M potassium phosphate buffer, pH 5.3, with or without 10^{-3} M 2,4-D. During the 12 hour treatment period the seedlings were placed in the dark at 21 to 22° C. After treatment, root tips (2 cm long) were cut from the seedlings and placed in Warburg vessels with 10^{-2} M potassium phosphate buffer, pH 5.3. Each vessel contained 7 root tips (about 180 mg fresh weight). To measure $C^{14}O_2$ production, 0.1 ml of C^{14}-substrate solution was added to each vessel. The vessels were shaken in the dark for 3.5 hours at 25° C, and the $C^{14}O_2$ released in respiration was collected in the KOH of the center well. Detailed experimental procedure is given in a previous communication (7).

For the measurement of substrate uptake, groups

of 14 root tips (2 cm long) were placed in 25 ml Erlenmeyer flasks which contained 4.2 ml of 10^{-2} M potassium buffer, pH 5.3. Two-tenths ml of radioactive substrate solution was added to each flask, and after thorough mixing, a 0.2 ml aliquot was removed for radioactivity measurement. The flasks were then stoppered and placed in a 25° C water bath. The flasks were shaken in the dark for 3.5 hours, after which a 2nd 0.2 ml aliquot was removed from each flask. The difference in the radioactivity of the 2 aliquots was used as a measure of the substrate taken up by the root tips. Aliquots containing carbon-14 labeled glucose and succinate were placed directly on planchets and, after the addition of absolute ethanol, were dried and counted. Aliquots containing carbon-14 labeled acetate were neutralized before they were plated.

The manometric experiments were conducted in the Warburg apparatus at 25° C. The vessels were shaken in the dark. For the measurement of oxygen uptake, the vessels were filled with air and 0.2 ml of 20 % KOH was added to the center well. The "direct method" (15) for the measurement of carbon dioxide evolution was used.

2,4-D (Eastman Kodak Co.) was neutralized with NaOH, and the sodium salt recrystallized twice from a water-alcohol solution. Radiochemicals were purchased from Volk Radiochemical Co. and Nuclear-Chicago Corp. The radioactive solutions were prepared to contain the following amounts in 0.1 ml: glucose, 4.4 micromoles, 40,000 counts per minute (cpm); sodium pyruvate, 2.0 micromoles, 40,000 cpm; sodium acetate, 1.3 micromoles, 70,000 cpm; sodium succinate, 1.3 micromoles, 50,000 cpm.

Results and Discussion

In preliminary experiments the effects of DNP on the metabolism of root tips from 2,4-D-treated and buffer-treated corn seedlings were studied by measuring the $C^{14}O_2$ produced from C^{14} labeled glucose and by measuring the respiratory gas exchange. The production of $C^{14}O_2$ by 2,4-D-treated root tips was less affected by DNP than was $C^{14}O_2$ production from buffer-treated root tips. At concentrations of DNP greater than 10^{-5} M, $C^{14}O_2$ production by buffer treated root tips was strongly inhibited. Appreciable inhibition did not occur using 2,4-D-treated root tips until concentrations of DNP greater than 5×10^{-5} M were employed. These results are shown in figure 1. A DNP concentration of 4.5×10^{-5} M was chosen for further studies, since at this concentration there was very little or no inhibition of $C^{14}O_2$ production from 2,4-D-treated root tips, but a strong inhibition (about 50 %) of $C^{14}O_2$ production from buffer-treated root tips. Furthermore, DNP at this concentration influenced the respiratory gas exchange of both the 2,4-D- and buffer-treated root tips in a qualitatively similar manner (table I); i.e., there was essentially no change in oxygen uptake and a large increase in CO_2 evolution. At higher concentrations of DNP respiratory gas exchange was inhibited (1), and at lower concentrations DNP affected the oxygen uptake of buffer-treated and 2,4-D-treated root tips to a different extent. Thus, at 3.4×10^{-5} M DNP the oxygen uptake of the buffer-treated root tips was increased about 33 %, and this increase became apparent within 30 minutes of adding DNP; while, with 2,4-D treated root tips, this concentration of DNP caused an increase of only 8 % in oxygen uptake, and the increase was not apparent until the 3rd hour of measurement.

Additional experiments were run using other C^{14}-labeled substrates. In all cases the production of $C^{14}O_2$

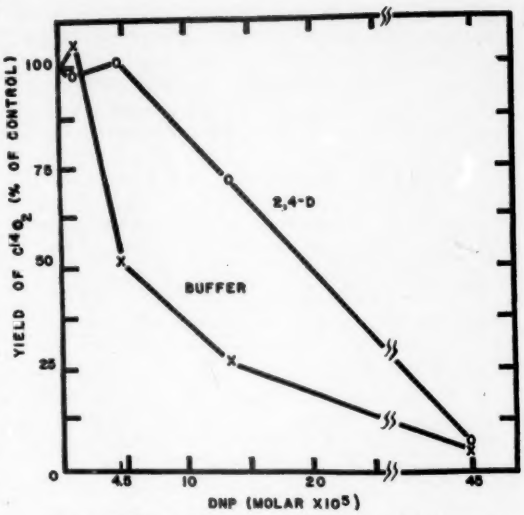

FIG. 1. Effect of DNP on the $C^{14}O_2$ production from uniformly labeled glucose by root tips from buffer-treated or 2,4-D-treated corn seedlings. Values given are in percent of $C^{14}O_2$ production from the control root tips to which no DNP was added. Treatment procedure is outlined in table I.

TABLE I

EFFECT OF DNP ON THE RESPIRATION RATES AND R.Q. OF ROOT TIPS FROM 2,4-D- AND BUFFER-TREATED CORN SEEDLINGS

TREATMENT*	Q_{O_2}**	Q_{CO_2}**	R.Q.	Q_{O_2}** +DNP	Q_{CO_2}** +DNP	R.Q.
Buffer	387	382	0.99	366	613	1.67
"	398	386	0.97	415	680	1.64
2,4-D + buffer	479	577	1.20	479	740	1.54
"	477	574	1.20	472	768	1.63

* Corn seedlings were placed with their roots in either 10^{-2} M phosphate buffer, pH 5.3, or buffer plus 10^{-3} M 2,4-D. Treatment was for 12 hours in the dark at 21 to 22° C. At the end of the treatment period 2 cm of the root tip was cut off. Root tips were weighed in groups of 7 and placed in Warburg vessels with 10^{-2} M phosphate buffer pH 5.3. DNP, when used, was added to the vessels to a final concentration of 4.5×10^{-5} M.

** Q_{O_2} and Q_{CO_2} values (μl per hour per gm fresh weight of tissue) are averages of the 1st 3.5 hours of measurement. Each value represents an average of the results from duplicate vessels.

was strongly inhibited by DNP (4.5×10^{-5} M) when buffer-treated root tips were used. When 2,4-D-treated root tips were used, however, there was not only an increased yield of $C^{14}O_2$ from all substrates (except glucose-6-C^{14}) but also DNP at this concentration had no inhibitory effect. These results are shown in table II.

TABLE II

EFFECT OF DNP ON THE PRODUCTION OF $C^{14}O_2$ FROM LABELED SUBSTRATES BY ROOT TIPS FROM 2,4-D- AND BUFFER-TREATED CORN SEEDLINGS

C^{14} substrate	Treatment*	% C^{14} added recovered as $C^{14}O_2$		% change due to DNP
		Control	+DNP**	
Glucose-U-C^{14}	Buffer	2.6	1.3	−50
"	2,4-D + buffer	4.8	5.3	+10
Glucose-1-C^{14}	Buffer	3.7	1.5	−59
"	2,4-D + buffer	6.6	7.4	+12
"	2,4-D + buffer	11.3	12.3	+ 9
Glucose-6-C^{14}	Buffer	4.6	0.9	−80
"	2,4-D + buffer	4.0	4.0	0
Pyruvate-1-C^{14}	Buffer	12.5	6.5	−48
"	2,4-D + buffer	18.3	19.7	+ 8
Pyruvate-2-C^{14}	Buffer	4.5	1.5	−67
"	2,4-D + buffer	12.3	14.0	+14
Pyruvate-3-C^{14}	Buffer	0.7	0.4	−43
"	2,4-D + buffer	6.1	5.6	− 8
Succinate-1-C^{14}	Buffer	6.3	1.5	−76
"	2,4-D + buffer	27.2	28.0	+ 3
Succinate-2-C^{14}	Buffer	1.5	0.6	−60
"	2,4-D + buffer	16.8	17.3	+ 3

* For treatment procedure see table I.
** DNP concentration, 4.5×10^{-5} M.

Three possible explanations as to why DNP inhibits $C^{14}O_2$ production from exogenous substrates are: 1) DNP increases the steady state concentration of endogenous metabolites, thus increasing the dilution of the entering labeled carbon; 2) DNP inhibits uptake of the labeled substrate by the root tips; 3) DNP inhibits "activation" (e.g., phosphorylation) of the substrates once they have entered the cells. The 1st explanation is a distinct possibility since DNP did cause a large increase in respiratory CO_2 (table I), which presumably resulted from an increase in the rate of glycolysis (1). An increase in the rate of glycolysis might result in or be caused by an increase in the concentration of glycolytic intermediates within the cell. Beevers (1) found that, at the concentration of DNP used in these experiments, corn roots accumulated acetaldehyde and alcohol, which perhaps would account for the inhibition of $C^{14}O_2$ production from uniformly labeled glucose (glucose-U-C^{14}), pyruvate-2-C^{14} and pyruvate-3-C^{14}, but would not account for the inhibition found with pyruvate-1-C^{14}.

The 3rd possible explanation, that DNP inhibits substrate "activation," is based on the known uncoupling effect of DNP on oxidative phosphorylation. Evidence is presented below which argues against this explanation of the DNP inhibition of $C^{14}O_2$ production. Nevertheless, this explanation remains attractive, though difficult to verify. Perhaps substrate "activation" is an integral part of the uptake mechanism, and thus no distinction between the two can be made in these experiments.

The 2nd possible explanation, that DNP inhibits substrate uptake, has some evidence to support it (1) and can easily be tested experimentally. If at zero time and after 3.5 hours, aliquots of the solution bathing the root tips are taken and their C^{14} content measured, the amount of C^{14} which entered the root tips can be measured. This method of measuring uptake of exogenous substrates has some drawbacks, 1) small differences between large numbers must sometimes be measured, 2) the uptake includes both the amount entering the cells and that entering the intercellular spaces and 3) the substrate is entering the roots from a continuously diminishing concentration of substrate in the bathing solution. In some cases, however, the decrease in concentration of the bathing solution was negligible and, in all cases, the same condition held for the $C^{14}O_2$ production experiments, allowing a direct comparison between uptake and $C^{14}O_2$ production (see below).

TABLE III

EFFECT OF DNP ON THE UPTAKE OF C^{14} LABELED SUBSTRATES BY ROOT TIPS FROM 2,4-D- AND BUFFER-TREATED CORN SEEDLINGS

C^{14} substrate	Treatment*	% uptake		% change due to DNP
		Control	+DNP**	
Glucose-U-C^{14}	Buffer	20.0	5.3	−74
"	2,4-D + buffer	14.0	8.4	−40
"	Buffer	26.7	1.9	−93
"	2,4-D + buffer	13.0	10.5	−19
"	Buffer	29.4	4.0	−86
"	2,4-D + buffer	12.6	7.0	−44
Acetate-1-C^{14}	Buffer	61.5	27.0	−56
"	2,4-D + buffer	80.0	84.4	+ 5
"	Buffer	59.0	22.1	−63
"	2,4-D + buffer	68.5	62.0	− 9
Succinate-1-C^{14}	Buffer	16.0	5.7	−64
"	2,4-D + buffer	33 7	20.8	−38
"	Buffer	15.9	4.7	−70
"	2,4-D + buffer	43.8	37.1	−15

* For treatment procedure see table I.
** DNP concentration, 4.5×10^{-5} M.

The results of the experiments of substrate uptake are shown in table III. When buffer-treated root tips were used, DNP strongly inhibited the uptake of the 3 substrates tested. Uptake of glucose and succinate by 2,4-D-treated root tips was inhibited also, but to much less an extent. Acetate uptake by the 2,4-D-treated root tips, on the other hand, was not inhibited by DNP. 2,4-D treatment, when compared to buffer treatment, caused a decrease in the amount of glucose and an increase in the amount of acetate and succinate taken up by the root tips.

From the results shown in tables II and III it is probable that inhibition of $C^{14}O_2$ production from the C^{14} substrates is primarily a reflection of the DNP inhibition of uptake of the substrates by the root tips. Apparently there were other sites of DNP inhibition, since $C^{14}O_2$ production from the 6th carbon of glucose

and the 2nd carbon of pyruvate was inhibited to a greater extent than from the other carbons of these compounds (table II). Inhibition of uptake can occur, however, without causing an inhibition of $C^{14}O_2$ production. Thus, a DNP inhibition of uptake of glucose and succinate by 2,4-D-treated root tips did not result in any inhibition of $C^{14}O_2$ production. Furthermore, 2,4-D treatment, although it decreased glucose uptake by approximately 50 %, resulted in nearly a doubling of $C^{14}O_2$ production. Apparently enough substrate entered the root tips, in these cases, to saturate the enzyme systems responsible for substrate oxidation. The increased $C^{14}O_2$ production observed when 2,4-D-treated root tips were used suggests a blocking of "synthetic" metabolic pathways by 2,4-D. Results of experiments in which $C^{14}O_2$ production from acetate-1-C^{14} and acetate-2-C^{14} were measured support the idea that a 2,4-D induced "synthetic block" ocurs. In comparison with buffer treatment, 2,4-D treatment caused an increase in $C^{14}O_2$ production from acetate; the percentage increase being much larger from acetate-2-C^{14} (approximately 700 %) than from acetate-1-C^{14} (approximately 300 %). DNP had essentially no effect on these results. In contrast to the results obtained with other substrates (table II), $C^{14}O_2$ production from acetate was increased by DNP when buffer-treated root tips were used. The increase from acetate-1-C^{14} was small, but amounted to 100 % from acetate-2-C^{14}. These results are shown in table IV.

The increase in CO_2 production from the methyl-carbon of acetate, which resulted either from 2,4-D treatment or the presence of DNP, is thought to be caused by a blocking of synthetic pathways leading from the Krebs cycle. Thus, if the flow of carbon atoms through the Krebs cycle was dissociated from other reactions of the cell except for the entrance of acetyl CoA and exit of CO_2, the ratio (rate of CO_2 production derived from the carbonyl carbon of acetyl CoA) / (rate of CO_2 production derived from the methyl carbon of acetyl CoA) would be unity (16). However, with tissues supplied with acetate-1-C^{14} and acetate-2-C^{14}, ratios considerably larger than unity were obtained, using tissues from both animals (16) and plants (C 1/C 2 ratio, table IV). High ratios are thought to result from a loss of the methyl carbon to synthetic pathways which originate at organic acids of the Krebs cycle. Blocking of these pathways results in an increase in CO_2 production from carbon that was originally the methyl carbon of acetate or acetyl CoA. Considering carbons 2 and 3 of pyruvate and carbons 1 and 2 of succinate as giving rise to or being derived from acetate, calculation of the above mentioned ratio can be made from the results obtained using pyruvate and succinate (table II). 2,4-D and DNP also caused a decrease in the ratios calculated from this data.

It is not necessary, however, to assume that acetate was metabolized via the Krebs cycle. Other schemes of acetate oxidation, that permit the methyl carbon to enter synthetic reactions while the carboxyl carbon goes to CO_2, could also be invoked to explain the results shown in table IV (e.g., see ref. (2)).

From the results obtained using labeled acetate, it is concluded that DNP also blocks synthetic pathways. An increased $C^{14}O_2$ recovery from glucose, pyruvate and succinate, which should result from such a block was not obtained, probably due to the inhibition of substrate uptake by DNP. Although acetate uptake by buffer-treated root tips was strongly inhibited when DNP was present, enough acetate entered the roots to saturate the enzyme systems responsible for acetate activation, and an increase in $C^{14}O_2$ production due to DNP was observed.

How do DNP and 2,4-D block synthetic pathways? DNP, since it is known to uncouple oxidative phosphorylation, might be expected to decrease synthetic reactions. However, it apparently did not decrease the rate of activation of acetate. The same can be said for 2,4-D; it decreased the uptake of glucose, but the glucose that entered the root cells was catabolised more rapidly. It is difficult to interpret these results entirely in terms of ATP level.

If indoleacetic acid (IAA) at a concentration of 10^{-3} M was substituted for 2,4-D in the above experiments, very similar results were obtained; i.e., an increased $C^{14}O_2$ production which was not inhibited by 4.5×10^{-5} M DNP and a low ($C^{14}O_2$ from acetate-1-C^{14}) / ($C^{14}O_2$ from acetate-2-C^{14}) ratio. Nance (11) has reported that in pea stem slices IAA (2.5×10^{-4} M) increased the incorporation of C^{14} from acetate-1-C^{14} into $C^{14}O_2$ and organic acids, and de-

TABLE IV

EFFECT OF DNP ON THE PRODUCTION OF $C^{14}O_2$ FROM ACETATE-1-C^{14} AND ACETATE-2-C^{14} BY ROOT TIPS FROM 2,4-D- AND BUFFER-TREATED CORN SEEDLINGS

TREATMENT*	% C^{14} ADDED RECOVERED AS $C^{14}O_2$			% C^{14} ADDED RECOVERED AS $C^{14}O_2$		
	Ac-1-C^{14}	Ac-2-C^{14}	C1/C2	Ac-1-C^{14} +DNP**	Ac-2-C^{14} +DNP**	C1/C2
Buffer	12.5	4.2	3 0	14.6	8.5	1.7
	13.5	3.5	3.9	15.8	8.3	1.9
	18.8	6.0	3.1	...	...	...
2,4-D + buffer	40.2	27.3	1.5	41.9	29.3	1.4
	43.2	31.6	1.4	38 4	27.0	1.4
	49.0	37.0	1.3	...	...	...

* For treatment procedure see table I.
** DNP concentration, 4.5×10^{-5} M.

creased the amount incorporated into pectic substances, lipides and water extractable materials. Thus, both 2,4-D and IAA seem to promote catabolism of exogenous substrates.

It appears that the principle difference in the action of DNP on 2,4-D-treated and buffer-treated root tips was on uptake of exogenous substrates. Why 2,4-D treatment should diminish or abolish the DNP inhibition of substrate uptake is not known. The action of DNP on plant metabolism is usually interpreted in terms of a change in the ATP/ADP ratio. However, our knowledge of the mechanism of substrate uptake by plant tissues is insufficient to evaluate this action of DNP in any but the most hypothetical terms. Johnson and Bonner (9) have reported that DNP inhibits uptake of 2,4-D by Avena coleptile sections. Since DNP, at a concentration of 4.5×10^{-5} M, had a similar effect on the respiration of both 2,4-D and buffer-treated root tips (table I), it is concluded that the decreased DNP inhibition of substrate uptake observed after 2,4-D treatment was not a result of an inhibition of DNP uptake or an inactivation of DNP.

In a previous communication from this laboratory (7), it was stated that 2,4-D treatment resulted in an increase in the amount of glucose catabolized via the pentose phosphate pathway, but did not affect the amount catabolized via the glycolytic pathway. This statement was made assuming that 2,4-D had no effect on glucose uptake. The results presented in this paper, however, show that 2,4-D treatment decreased glucose uptake by approximately 50 %. Despite this decrease in glucose uptake, the $C^{14}O_2$ recovered from glucose-6-C^{14} was decreased little, if any, by 2,4-D treatment (table II and ref. (7)). If the 6th carbon of glucose enters the Krebs cycle entirely via the glycolytic pathway, then it must be concluded that the amount of glucose catabolized via the glycolytic pathway also was increased by 2,4-D treatment. It is, of course, quite possible that more than one pathway was contributing to the yield of $C^{14}O_2$ from glucose-6-C^{14}.

2,4-D treatment caused an increase in the respiratory quotient (R.Q.) in the absence of DNP (table I). Previous studies with whole seedlings of pea and corn showed no increase in R.Q. as a result of 2,4-D treatment (8). It is not known whether this difference was due to metabolic changes following excision or to masking of the gas exchange of the root tip portion of the seedling by the gas exchange of the whole seedling.

Summary

1. DNP, at a concentration of 4.5×10^{-5} M, inhibited $C^{14}O_2$ production from labeled glucose, pyruvate and succinate, when root tips from buffer-treated corn seedlings were used. No inhibition was found, however, when root tips from 2,4-D-treated seedlings were used.

2. Uptake of the labeled substrates by buffer-treated root tips was strongly inhibited by DNP. Pretreatment with 2,4-D, however, caused a diminution or abolishment of the DNP inhibition of substrate uptake.

3. DNP and 2,4-D each increased $C^{14}O_2$ production from acetate-1-C^{14} and acetate-2-C^{14}; the percentage increase being larger from the methyl carbon than from the carboxyl carbon.

4. It is concluded that both 2,4-D and DNP promote catabolism of exogenous substrates by blocking synthetic metabolic pathways.

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EFFECT OF SOME SUBSTITUTED BENZOIC ACIDS AND RELATED COMPOUNDS ON THE DISTRIBUTION OF CALLUS GROWTH IN TOBACCO STEM EXPLANTS [1, 2]

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It has been observed in this laboratory (15) that the normally polar distribution of callus growth on tobacco stem explants becomes non-polar in the presence of low levels of 2,3,5-triiodobenzoic acid (TIBA), while the amount of callus growth is not diminished. It was thought that this phenomenon might reflect a specific inhibition by TIBA of the polar transport of auxin. Subsequent studies (10, 11, 13) were carried out to test this hypothesis. TIBA was found (13) to inhibit transport of indole-3-acetic acid (IAA) in the classical Went (23) transport test and in tobacco stem segments as determined directly by chemical and bio-assay of IAA previously introduced into the plants and then extracted from the apical and basal portions of the stem segments. Other compounds found to cause a redistribution of callus growth and also to inhibit transport in the Went assay were 2-bromo-3,5-dichlorobenzoic acid, γ-phenylbutyric acid, 2,4-dichlorophenoxy acetic acid (2,4-D), and phenylacetic acid (10). The inhibitory effects on polar IAA transport by TIBA and 2,4-D have been independently confirmed by Hay (4) and by Zwar and Rijven (24). Another instance of apparent inhibition of IAA transport by TIBA has been reported by Kuse (6), who measured axillary bud growth and petiole extension in Ipomoea explants (consisting of a section of stem containing 1 node and its leaf) after 7 days' growth in nutrient solution. Bud growth was inhibited by the intact leaf, or by IAA applied to the cut end of a debladed petiole. TIBA applied midway along the petiole permitted bud growth on explants having either auxin source. The portion of the petiole distal to the TIBA was reported to have grown more than the proximal part. Kuse suggested that both effects resulted from accumulation of auxin above the point of TIBA treatment, as though transport had been prevented.

Because two substituted benzoic acids had been shown to prevent auxin transport at concentrations less than those required to inhibit growth, and because these compounds have been studied extensively for auxin activity (8, 9, 20), it was thought to be of interest to screen a number of them for this apparently specific effect on polar transport, as shown by their influence on the growth and distribution of callus on tobacco stem explants.

As two of the TIBA analogs (2-hydroxy- and 2-amino-3,5-diiodobenzoic acids) have been found to uncouple phosphorylation in animal tissue homogenates (H. A. Lardy, personal communication), it was decided to test also 2 other highly active uncouplers, 2,6-dibromo-4-nitrophenol, and 3,5-diiodo-L-tyrosine.

In an attempt to improve the sensitivity of the method of Niedergang-Kamien and Skoog (13) for testing transport inhibition by direct chemical analysis of the distribution of added auxin in treated and untreated tobacco stem sections before and after transport, the distribution of added IAA was measured after a 2-hour transport interval.

Although many experiments (10, 11, 12, 13) indicate that TIBA inhibits the polar movement of IAA, they also show that TIBA drastically reduces the level of extractable IAA, which complicates the assay. The reduction was so great that even with chromatographic purification, not enough added IAA was recoverable from TIBA-treated tobacco stem tissue to permit colorimetric assays. For this and other reasons it was desirable to test the effect of TIBA on the movement of radioactive IAA, the distribution of which in the tissue could be determined without extraction.

METHODS AND MATERIALS

Stem tissue of *Nicotiana tabacum* L. var. Wisconsin 38 was cultured in 125-ml Erlenmeyer flasks each containing 50 ml of medium, as described by Niedergang-Kamien and Skoog (13). The external portion was stripped off down to the cambium in order to expose aseptic tissue; thus the cultured pieces contained part of the cambium, xylem, internal phloem, and pith. Into each flask was placed either a single cylinder or 3 segments obtained by cutting the cylinders tangentially.

The basal medium was the modified White's mineral salts-sucrose-agar medium described by Skoog and Tsui (16). The concentration of KH_2PO_4 was tripled (37.5 mg/l), and the iron source was NaFe-Sequestrene (25 mg/l NaFe-ethylenediaminetetraacetic acid). The chemicals to be tested were prepared as alkaline 7.5×10^{-3} M stock solutions, and diluted to give final test concentrations of 5×10^{-3}, 1×10^{-4}, 1×10^{-5}, and 1×10^{-6} M. Each diluted solution was adjusted to pH 6.1 with HCl before autoclaving. To detect auxin inhibitors, the 1×10^{-4} and 1×10^{-5} M concentrations were tested also in the presence of 1×10^{-5} M IAA, and in most experiments cultures supplied with 2×10^{-5} M TIBA were included as checks. In order to avoid thermal decomposition, the 2,5-diiodobenzoic acid and 2,4-dihydroxybenzoic acid were filter-sterilized and added aseptically to the autoclaved basal medium.

As a standard procedure, 7 replicate flasks with segments and 8 flasks with cylinders were used for each concentration of the test compound. Observa-

[1] Revised manuscript received July 31, 1958.
[2] This study is part of the Ph D thesis work of GWK Jr.
[3] Present address: Department of Botany, University of Michigan, Ann Arbor.

tions were made periodically and sample tissue pieces were photographed after 3 to 8 weeks. A record was made of the amount and distribution of callus growth, and the amount of pith enlargement obtained. Cylinders sometimes reveal subtler effects on polarity than do segments (13); however, in the present work, the cylinders yielded no information not also observed in the segments, hence only the latter are illustrated. The effects of active chemicals on callus growth and distribution were striking, and were clearly seen in visual comparisons between treatments, as is evident from figure 3. Data for inactive compounds are summarized here in tabular form only; detailed photographic records of all experiments are found in (5).

The distribution of added IAA in tobacco stem sections after transport was determined by a modification of the method of Niedergang-Kamien and Skoog (13). Tobacco stems, decapitated and stripped of all but the upper 3 well-expanded leaves, were placed in a large beaker containing a 10 mg/l solution of IAA, set before an electric fan, and uptake via the transpiration stream was allowed for 1 hour. The stems were then cut into 1-cm sections and placed in a moist chamber for 2 hours to permit transport. The sections rested across shelves made of two 3-mm glass rods cemented together at the ends. This arrangement permitted the sections to touch the glass tangentially at only 2 points and thus prevented formation of a continuous moisture film between the glass and the tissue which might serve as a pathway for auxin diffusion. After transport, the apical half of each segment was removed and cut into 5 parts. These were pooled and extracted for 3 hours with ether. The basal half was cut into 1-cm sections which were pooled separately by position and also extracted as above. The extracts were chromatographed in isopropanol : ammonia : water, 10 : 1 : 1 (18), eluted with water, and the concentration of IAA in the eluate determined colorimetrically with the $FeCl_3$-$HClO_4$ modified Salkowski reagent (2).

The experiments with IAA labelled in the methylene carbon with C^{14} were carried out as follows. Seedlings of *Phaseolus vulgaris* L. var. Kentucky Wonder were grown in the light for 6 to 8 days. From each hypocotyl a 2-cm section was removed, the apical end being 5 mm below the cotyledonary node. The apical half of the section was marked with an India ink dot applied 3 mm apically from the center. Forty sections per treatment were immersed in a layer of solution about 2 mm deep (4 ml in a 5-cm Petri dish) and uptake was permitted for 3 hours. To avoid complications from surface contamination and handling, the sections were rinsed upon removal from the solution and blotted dry, and a 5-mm section was removed from each end, so that only the central 1-cm portion was used for measurements. Half of the 1-cm sections were cut at once into 2-mm lengths, and half were placed in a moist chamber for 2 hours to permit transport before cutting. In the moist chamber the tissues were placed on glass rod shelves as described above for the tobacco experiment. After cutting, the sections from each position (apex to base) were pooled in a vial.

In early experiments the sections were simply dried, placed in a planchet, and counted whole. Although statistically valid results were obtained which showed that transport had occurred in the controls but not in the presence of TIBA (5), the changes were not great, and the variance was relatively high. An improved technique consisted of adding 0.2 ml of 20 % KOH to each vial and macerating the fresh sections with a glass rod. Then several drops of CS_2 were added and further trituration performed. The macerate was kept at room temperature overnight while the xanthate formed. This procedure solubilized the cellulose to a large degree and gave an adequately homogeneous material for plancheting on stainless steel cupped planchets. After drying in an oven the planchets were counted with a mylar-window gas flow counter. The control solution contained 31 μg/ml of IAA-α-C^{14} having a specific activity of 0.9 mC/mM. This gave the solution an activity of ca. 3.66×10^5 disintegrations/min/ml. The test solution contained in addition 5×10^{-4} M TIBA. In the experiment described below, 3 replicate dishes of each solution were used.

Results and Discussion

It has previously been shown (10, 13) that added IAA is transported to the basal halves of 1-cm tobacco stem segments in 3 hours, in quantities many times the endogenous level. It was necessary to shorten the transport interval for reasons of convenience, so an experiment was performed to measure the distribution after 2 hours to see if measurable transport had occurred. Figure 1 shows that nearly complete transport

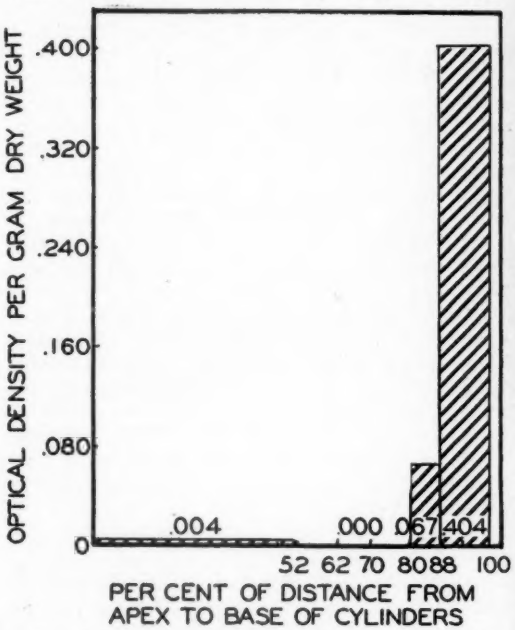


Fig. 1. Distribution of added IAA extracted from different portions of 1-cm tobacco stem segments after 2 hours of transport. The distance along the abscissa is proportional to the dry weight of the tissue in each position as the cuts were not precisely spaced.

of the IAA to the basal 2 mm had occurred. The optical densities were not converted to μg-equivalents of IAA before graphing in this experiment because it was found later that one-dimensional chromatography did not remove all color test inhibitors; however, the purpose of the experiment was primarily to show the distribution. An approximate minimum value based on standards run at the same time is 4 μg/g dry wt. To determine if continuous auxin production occurs in cultured tobacco stem sections, the basal ends of 2 groups of peeled segments were removed after 3 hours; from 1 group, an additional basal portion was removed after 6 hours. All the pieces were subsequently cultured for 36 days. The results are shown in figure 2. If callus growth depended solely on endogenous auxin initially present, one would expect callus growth only on the basal ends which were cut off, as they should have contained all the auxin after 2 to 3 hours. However, callus growth occurred on all pieces, and the amount on the pieces cut after 6 hours was no less than that on the pieces removed after 3 hours. These results argue for a continuous auxin synthesis in the cultured tissues. Therefore any compound which causes apolar callus distribution but does not inhibit callus growth may interfere rather specifically with the transport of this endogenous auxin. Because it is also possible that callus redistribution results from an auxinic action of the non-transported test chemical itself, confirmatory experiments of a more direct nature are needed for full interpretation.

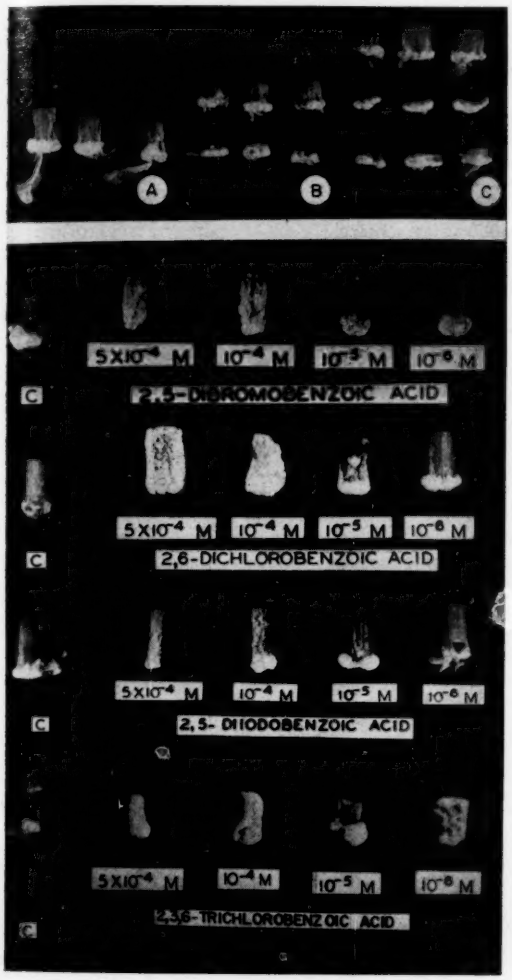


FIG. 2 (*top*). Callus growth on tobacco stem segments with basal ends removed at successive intervals, subsequently cultured 36 days. A: Intact controls. B: "Parent pieces" (upper) and basal ends (lower) removed after 3 hours. C: "Parent pieces" (upper), basal ends removed after 6 hours (center), and basal ends removed after 3 hours (lower). All pieces are oriented with basal ends toward bottom of photograph; basal ends are not necessarily matched to respective "parent pieces".

FIG. 3 (*bottom*). Effect of concentration of 4 benzoic acid derivatives on the distribution of callus growth in tobacco stem segments cultured in vitro. C (left): Controls with polar callus growth (at basal ends). Age of cultures: 37, 47, 42, and 45 days, respectively, top to bottom.

As shown in figure 3 and table I, 2,3,6-trichlorobenzoic acid, 2,6-dichlorobenzoic acid and 2,5-dibromobenzoic acid were highly active in causing apolar distribution of callus growth, and 2,5-diiodobenzoic acid was weakly active in this respect. The 1st 3 com-

TABLE I

ACTIVITY OF SOME SUBSTITUTED BENZOIC ACIDS AND RELATED COMPOUNDS IN CAUSING APOLAR DISTRIBUTION OF CALLUS GROWTH IN TOBACCO STEM SEGMENTS CULTURED IN VITRO.*

Strongly active	
2,5-dibromobenzoic**	2,6-dichlorobenzoic**
2,3,6-trichlorobenzoic***	
Weakly active	
2,5-diiodobenzoic**	
Inactive	
2-bromobenzoic**	2-chloro-4-aminobenzoic†
3-bromobenzoic**	2-chloro-5-nitrobenzoic†
4-bromobenzoic**	2,4-dimethoxybenzoic†
2-iodobenzoic	2,5-dinitrobenzoic
3-iodobenzoic	2,4-dihydroxybenzoic
4-iodobenzoic	2,5-dihydroxybenzoic
2-nitrobenzoic	2-amino-3,5-diiodobenzoic
4-nitrobenzoic	2-hydroxy-3,5-diiodobenzoic
2-acetylaminobenzoic	2-hydroxy-3,5-dinitrobenzoic
4-acetylaminobenzoic	2,4-dichloroanisole
2,4-dichlorobenzoic	3,5-diiodo-L-tyrosine†
3,4-diiodobenzoic**	2,6-dibromo-4-nitrophenol†
potassium iodide††	

* Source of chemicals: Eastman White Label except as indicated below.

** Kindly supplied by Dr. R. L. Weintraub, Ft. Detrick, Maryland.

*** Heyden Chemical Co.

† Kindly supplied by Prof. H. A. Lardy, Enzyme Institute, University of Wisconsin (originally from commercial sources).

†† Mallinckrodt Analytical Reagent.

pounds did not inhibit callus growth, whereas the less active 2,5-diiodobenzoic acid did so markedly. Neither 2,3,6-trichlorobenzoic, 2,6-dichlorobenzoic, nor 2,5-dibromobenzoic acid alone had any visible effect on pith enlargement; 2,6-dichlorobenzoic and 2,5-dibromobenzoic acids failed to affect the pith enlargement caused by added IAA. Because the amount of 2,5-diiodobenzoic acid was limited, this compound was not tested in the presence of added IAA; 2,3,6-trichlorobenzoic acid was not tested with added IAA because insufficient plants were available at the time.

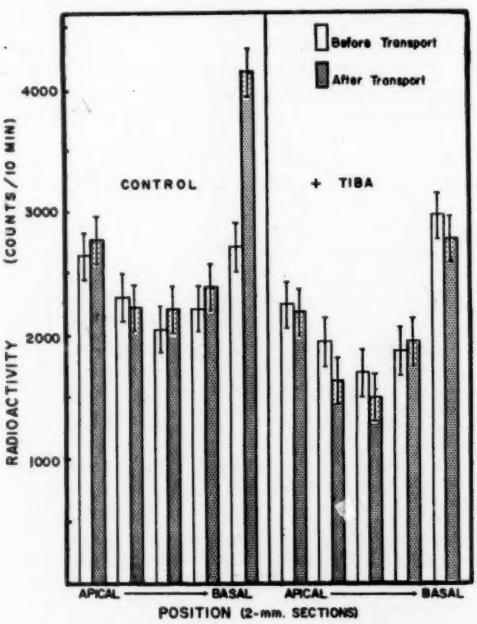
The remaining compounds listed in table I had no effect on callus distribution. As a check on possible effects of the high potassium levels introduced as the excess base used in dissolving the test compounds, and of any iodide resulting from TIBA breakdown, potassium iodide was tested at levels as high as 10^{-3} M. No effect on callus distribution was observed; only a slight inhibition of callus growth resulted, and that only at the highest level of KI tested.

It is noteworthy that several of the substances tested here which are known to be phosphorylation uncouplers did not alter the typically polar distribution of callus growth, although they did severely inhibit the amount. Earlier reports (1, 12) that such uncouplers did inhibit auxin transport in the Went assay, therefore, may reflect a non-specific inhibition due to a general toxic effect, rather than a selective action on polarity. The uncoupling agents did markedly inhibit the cell enlargement of the pith tissue which occurs in the presence of added IAA (table II). The relative inhibition is indicated by the number of (–)

TABLE II

EFFECT OF THE COMPOUNDS LISTED IN TABLE I ON PITH ENLARGEMENT OF TOBACCO STEM TISSUE CULTURED IN VITRO IN THE PRESENCE OF 10^{-5} M IAA

Inactive at 10^{-4} and 10^{-5} M (0)	
3-bromobenzoic	2,6-dichlorobenzoic
4-bromobenzoic	2,5-dibromobenzoic
3-iodobenzoic	2,5-dinitrobenzoic
4-iodobenzoic	2-chloro-5-nitrobenzoic
2-nitrobenzoic	2,4-dihydroxybenzoic
4-nitrobenzoic	2,5-dihydroxybenzoic
4-acetylaminobenzoic	2-hydroxy-3,5-dinitrobenzoic
2,4-dichloroanisole	
Inhibitory at 10^{-4} M, inactive at 10^{-5} M (—)	
2-bromobenzoic	2-acetylaminobenzoic
2-iodobenzoic	2,4-dichlorobenzoic
2,4-dimethoxybenzoic	
Strongly inhibitory at 10^{-4} M, somewhat at 10^{-5} M (– –)	
2-chloro-4-aminobenzoic	2-amino-3,5-diiodobenzoic
2,3,5-triiodobenzoic	3,5-diiodo-L-tyrosine
Strongly inhibitory at both 10^{-4} and 10^{-5} M (– – – –)	
3,4-diiodobenzoic	2-hydroxy-3,5-diiodobenzoic
2,6-dibromo-4-nitrophenol (– – – – –)	



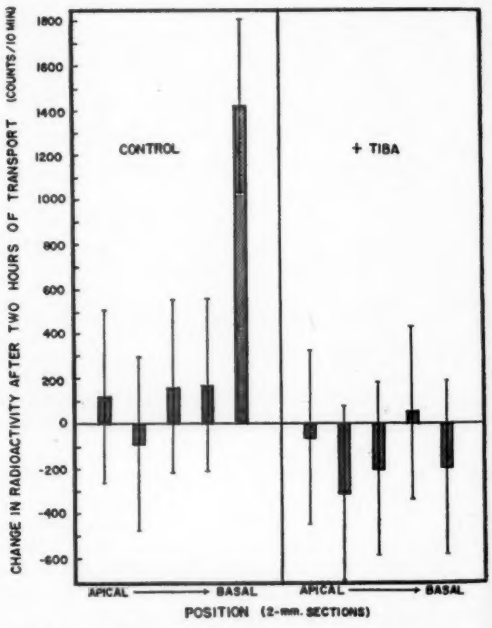


FIG. 4 (*left*). Effect of TIBA on the distribution of IAA-*a*-C^{14} in bean hypocotyl sections before and after a transport interval. The clear bars show the distribution before transport; the shaded bars, after. The vertical lines at the top of each bar indicate the 95 % confidence limits.

FIG. 5 (*right*). Change in radioactivity of different portions of 1-cm bean hypocotyl sections after 2 hours of transport of IAA-*a*-C^{14}. The data are derived from those of figure 4 by difference.

signs and is based on the extent of inhibition at the 2 levels tested. Differences within each group are not considered important, except that 2,6-dibromo-4-nitrophenol appeared to be the most toxic of all the compounds tested.

It is of interest that the 2-bromo- and 2-iodo- derivatives were stronger growth inhibitors than their 3- and 4-substituted isomers, which is consistent with the view of Muir and Hansch (8) that displacement of an electron-attracting substituent in one of the *ortho* positions is involved in the growth reaction induced by benzoic acids. The disubstituted benzoic acids with substituents in the 4-position were growth-inhibiting, except 2,4-dihydroxybenzoic acid. Veldstra (21) has reported that substituents larger than fluorine in the 4-position rendered the benzoic acids inhibitory or inert with respect to coleoptile elongation or pea stem curvature.

Preliminary experiments in which other criteria were used to test for transport inhibition were performed with 2,6-dichloro- and 2,5-dibromobenzoic acids. The results were in agreement with the culture experiments. These tests included the release of apical dominance in bean seedlings, and the direct measurement of IAA extracted from tobacco stem sections before and after transport.

The results of a typical experiment to test the effect of TIBA on the distribution of IAA-*a*-C^{14} in bean hypocotyl sections before and after transport are shown in figures 4 and 5. In figure 4 is shown the total activity in 2-mm sections from each position, before and after transport, and the 95 % confidence limits. The significant feature is the increase in radioactivity in the basal 2 mm of the control sections after transport, and the lack of any such increase in the presence of TIBA. This difference is emphasized in figure 5, in which the activity before transport has been subtracted from that after. No significant change occurred in the TIBA-treated tissue, whereas marked transport occurred in the controls.

Figure 4 also shows that less radioactivity was found in the central 2-mm portion than at the ends, in both treated and control populations. Two possible explanations are: 1), that auxin destruction at the cut surfaces causes non-transportable breakdown products to accumulate at the ends, or 2), that more auxin can enter the tissue via the cut ends than via the epidermis. Although auxin destruction at cut surfaces is a well-known phenomenon (17), the time for such action was limited because in the "before transport" treatment the cut ends which had been in contact with the solution were removed immediately before the central 1-cm sections were cut into 2-mm lengths. Even so, the counts were higher in the apical portion than in the central ones. The second alternative is supported by the findings of Thimann and Schneider (19) that IAA enters Avena coleoptiles more rapidly through cut surfaces than through the epidermis. However, the subject of differential permeability has been much discussed (14, 19) and some indirect evidence has been presented for increased permeability at the intact side of slit pea stems (14).

Various studies have established that the polar auxin transport mechanism expends metabolic energy (1, 3, 22) and is, in fact, inhibited by compounds which interfere with respiration or energy transfer. These findings have been reviewed and extended by Niedergang-Kamien and Leopold (12), who report that polar auxin transport can also be inhibited by certain sulfhydryl-binding compounds at concentrations which do not inhibit growth or respiration. They suggest that the inhibition of transport is of a more specific type. Because TIBA has been reported to bind sulfhydryl groups (7), and other sulfhydryl reagents have been shown to inhibit auxin transport (12), the action of TIBA on transport may indeed involve sulfhydryl binding. However, a number of other auxin-like compounds have been found to inhibit auxin transport. In addition to those reported by Niedergang-Kamien and Skoog (13), and those described here, Zwar and Rijven (24) have reported that 2,4-dichlorophenoxypropionic, 2,4-dichlorophenoxybutyric, 2,4,6-trichlorophenoxyacetic, and indole-3-propionic acids inhibit the transport of IAA through bean hypocotyl segments. Compounds found inactive by Zwar and Rijven were *p*-chlorophenylisobutyric acid, 2,4-dichloroanisole, coumarin, *o*-isopropyl-N-phenylcarbamate, indole, indole-3-acetonitrile, and phenylacetic acid.

Although supporting experiments (e.g., direct analyses) are needed to establish the point, the effects on callus growth and distribution of 2,6-dichlorobenzoic acid, 2,5-dibromobenzoic acid, and 2,3,6-trichlorobenzoic acid so closely resemble those of TIBA as to suggest that inhibition of auxin transport is involved in all cases. The growth of callus indicates that concentrations of these compounds which stop polar distribution do not interfere with respiration or energy transfer. Although further work is needed to determine if these auxin-like transport inhibitors also involve sulfhydryl binding or function by other mechanisms, the high degree of structural specificity found in the experiments reported here suggests that auxin transport has a stereochemical aspect, perhaps involving competition for "carrier" or "transport" sites, as has been postulated in a general sense by Niedergang (10) and by Zwar and Rijven (24) to account for the facts 1) that so few auxins are actively transported, and 2) that none has been found to be transported nearly as rapidly as IAA.

Summary

The capacity of tobacco stem segments to transport polarly many times the endogenous levels of IAA was shown by the distribution in segments after 2 hours of transport. Presumptive evidence for continuous auxin production and transport by cultured tobacco stem explants was obtained.

Twenty-nine compounds, mostly substituted benzoic acids, were tested on tobacco stem explants for their effect on callus growth and distribution. Three compounds, 2,3,6-trichloro-, 2,6-dichloro- and 2,5-dibromobenzoic acids, were highly active in causing apolar distribution of callus growth, and 2,5-diiodobenzoic

acid was weakly active. The remaining 25 compounds were inactive with respect to callus distribution.

When tested alone, the highly active compounds had no inhibitory effect on callus growth; 2,5-diiodobenzoic acid was markedly inhibitory.

None of these compounds alone noticeably stimulated pith cell enlargement. Neither 2,5-dibromo- nor 2,6-dichlorobenzoic acid affected the amount of pith cell enlargement obtained in response to a stimulatory IAA level (10^{-5} M); 2,3,6-trichloro- and 2,5-diiodobenzoic acids were not tested together with IAA.

TIBA was found to inhibit the polar transport of IAA-*a*-C^{14} in bean hypocotyl sections, thus confirming earlier studies by a method not dependent on extraction or bio-assay of IAA.

This work was supported in part by a research grant to F. Skoog from the American Cancer Society. The authors are grateful to Prof. A. J. Riker of the Dept. of Plant Pathology for his continued interest and for an assistantship on his project funds from the Wisconsin Alumni Research Foundation, granted by the University Research Committee of the Graduate School. They are indebted to Mr. Langdon P. Divers for some of the photographs, and to Prof. A. G. Norman, Dept. of Botany, University of Michigan, for enabling them to test 2,3,6-trichlorobenzoic acid and for suggesting the maceration technique used in the C^{14} experiment. Some of the experiments with IAA-*a*-C^{14} were done with a sample kindly supplied by Prof. J. P. Bennett of the University of California, Berkeley; the others, with a commercial sample from Tracerlab, Boston.

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DARK FIXATION OF CO_2 BY TOBACCO LEAVES [1, 2, 3]

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The ability of non-succulent plants to fix CO_2 in the absence of light has been previously demonstrated by several investigators (*2*, 5, 11, 17, 21). However, the succulents have received the greatest attention because of the large amounts of CO_2 incorporated into organic acids in the dark.

Although these earlier reports strongly suggested that dark fixation of CO_2 is an ubiquitous phenomenon in plants other than succulents, the nature of the biochemical reactions involved in this process has not been critically examined. Concurrently with the studies carried out in our laboratory on succulent metabolism (12, 13) we have investigated non-succulent dark CO_2 metabolism in *Nicotiana tabacum*. Studies concerning the nature of the initial carboxylation reaction and the fate of the fixed $C^{14}O_2$ during the subsequent metabolic reactions in the absence of light were carried out with excised tobacco leaves. These studies show that the pathways for the dark metabolism of CO_2 by succulent and non-succulent leaves appear to be fundamentally the same.

MATERIALS AND METHODS

The methods used in the study of the dark fixation of CO_2 in excised tobacco leaves are the same as those described in detail in earlier communications (12). *Nicotiana tabacum* (var. Hicks) plants used in these experiments were grown at the Earhart Plant Research Laboratory, Division of Biology, California Institute of Technology, Pasadena, California and most generously supplied by Dr. H. R. Highkin. Immediately before use, approximately 1 g of young leaves (5 to 7 cm long) were taken from the apex of the plants. The leaves were placed in an apparatus which permits exposure to $C^{14}O_2$ in total darkness. After equilibration of the leaves for 5 minutes in the dark to remove any transient reducing compounds of photosynthesis, $C^{14}O_2$ generated from 5.0 mg $BaC^{14}O_3$ (specific activity 120 μc/mg) was admitted into the chamber. After suitable exposure to $C^{14}O_2$, the reaction was terminated by homogenization in boiling 80 % ethanol.

The ethanol homogenate was filtered, the filtrate extracted with Skellyslov A, and the extract concentrated to a volume of 3 ml under reduced pressure. Concentrated alcoholic extracts were separated by two dimensional paper chromatography, phenol (80) : water (20), (w/w) in the 1st direction and *n*-butanol (74) : acetic acid (19) : water (50), (v/v/v) in the 2nd direction. Compounds were located with the appropriate sprays: amino acids with ninhydrin in collidine spray of Levy and Chung (9), organic acids with a mixed indicator spray of 3 g brom-phenol blue and 1 g methyl red per liter of 95 % ethanol. Radioactive compounds were located by radioautograms made by exposing the chromatograms to "no-screen" x-ray film. Activity of each compound was measured directly on the paper using an end-window Geiger tube.

2,4-Dinitrophenylhydrazones were prepared according to a modified procedure described by Ranson[4], in order to trap the α-keto acids which would otherwise be lost during the preparation and the subsequent chromatographic analysis of the plant extract. Approximately 8 g of leaves, with the midribs removed, were exposed to $C^{14}O_2$ in the absence of light for 10 minutes. The reaction was stopped by homogenization of the leaves with 20 ml of ice cold 5 *N* H_2SO_4. The homogenate was immediately filtered and the residue washed with 5 ml of 5 *N* H_2SO_4. Twenty-five ml of a saturated solution of 2,4-dinitrophenylhydrazine in 5 *N* H_2SO_4 was then added to the filtrate and placed in the cold for 24 hours to permit the formation of the 2,4-dinitrophenylhydrazones. The hydrazones were extracted from the acid solution with three 30-ml portions of ethyl acetate. The ethyl acetate was then extracted with three 30-ml portions of ice cold 10 % Na_2CO_3. The combined 10 % Na_2CO_3 extracts were adjusted to pH 1.0 with ice cold 5 *N* H_2SO_4 and extracted with three 20-ml portions of ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous Na_2SO_4 and concentrated to a volume of 2 ml under reduced pressure. The 2,4-dinitrophenylhydrazone derivatives were chromatographed on buffered paper by the method of Isherwood and Cruikshank (7) and identified by co-chromatography with authentic samples. Radioautograms were prepared as above. The activity of each compound was measured directly on the paper using an end-window Geiger tube.

The enzyme, phosphoenolpyruvate carboxylase, was prepared from acetone powder of tobacco leaves and assayed by the method described by Bandurski and Greiner (1). The acetone powder was prepared by homogenizing 50 g of tobacco leaves with 500 ml of acetone in a Waring blendor at —20° C. The homogenate was immediately filtered through a Buchner

[1] Received August 7, 1958.

[2] This investigation was supported by a research grant from the Tobacco Industry Research Committee. The facilities of the Allan Hancock Foundation were generously provided.

[3] A preliminary report was presented at the meeting of the American Society of Plant Physiologist, August 26–30, 1956, University of Connecticut, Storrs, Connecticut.

[4] Personal communication.

funnel and washed with 50 ml of —20° C acetone. The powdered residue was dried over PCl_5 under vacuum. The dried acetone powder was stored in a tightly sealed bottle at 0° C. The enzyme was prepared by extracting 0.2 g of the acetone powder with 5.0 ml of 0.005 M pH 7.5 TRIS buffer, in the cold. After centrifugation this extract was used directly.

Results and Discussion

Exposure of the tobacco leaves to $C^{14}O_2$ in the dark for periods from 6 seconds to 5 hours, resulted in the sequential incorporation of the radioactive carbon into several compounds, principally organic and amino acids. Table I shows the total amount of radioactivity

Table I

Rate of the Dark Fixation of $C^{14}O_2$ By Leaves of Nicotiana, Tabacum and Bryophyllum Calycinum

Time dark exposure to $C^{14}O_2$ (min)	cpm/mg of leaves/mg $BaCO_3$*	
	N. tabacum	B. calycinum
1	9	6
5	22	10
15	50	23
30	79	109
60	124	407
120	209	2,280

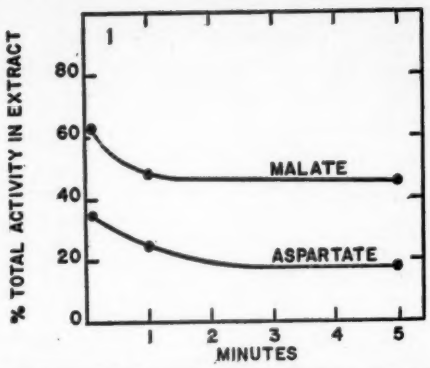
Leaves of tobacco and Bryophyllum were exposed independently to $C^{14}O_2$ in the dark for the indicated time intervals. The reaction was terminated by homogenizing with boiling 80 % ethanol, and an aliquot of the homogenate assayed for radioactivity.

* Samples were counted within a statistical error of 5 %.

incorporated by tobacco and Bryophyllum leaves exposed to $C^{14}O_2$ in the dark for various intervals. It appears that tobacco can fix CO_2 more rapidly in the initial stages of the dark carboxylation reaction. However, after the 1st 15 minutes the rate in the Bryophyllum seems to surpass that found in the tobacco leaves.

Malate and aspartate were the major detectable radioactive products on chromatograms of tobacco leaves exposed to $C^{14}O_2$ for 6 seconds. However, traces of citrate were also observed. The amount of citrate accounted for less than 0.01 % of the total activity present. The percent of total activity found in malate and aspartate after successively longer periods of exposure to $C^{14}O_2$ are plotted in figure 1. The extrapolation of these values to zero time strongly suggests the presence of a common precursor to malate and aspartate, probably oxaloacetate. To test this hypothesis, oxaloacetate was isolated and identified by cochromatography as the 2,4-dinitrophenylhydrazone derivative from tobacco leaves exposed to $C^{14}O_2$ in the dark for 10 minutes. It contained considerable radioactivity.

Since these results are similar to those found for succulent leaves (12, 13), the enzyme mediating the initial fixation of CO_2, phosphoenolpyruvate carboxylase, was extracted, and the data from a typical en-



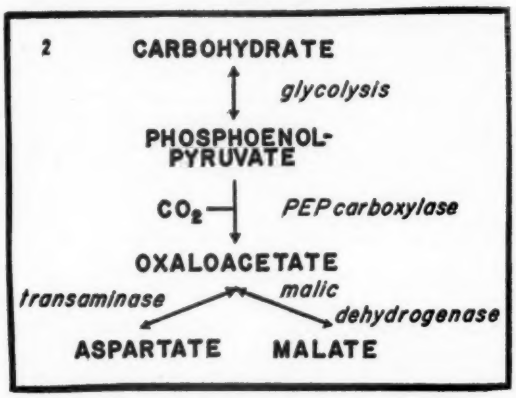


Fig. 1. Radioactivity of malate and aspartate expressed as percent total activity in extract as a function of time.

Fig. 2. Initial reactions involved in the dark fixation of CO_2 by tobacco leaves.

zymatic assay are presented in table II. From these results it would appear that the initial carboxylation reaction is identical in both *Nicotiana tabacum* and *Bryophyllum calycinum*. It is suggested that figure 2 represents the initial reactions involved in the dark

Table II

Phosphoenolpyruvate Carboxylase Activity in TRIS Buffer Extracts of Acetone Powder Prepared from Tobacco Leaves

Condition	cpm fixed in reaction mixture
Enzyme + phosphoenolpyruvate	10,600 ± 530
Enzyme — phosphoenolpyruvate	400 ± 20
Boiled + phosphoenolpyruvate enzyme	0

Each tube contained 60 micromoles TRIS hydrochloride pH 7.5, 20 micromoles $MgSO_4$, 100,000 cpm $NaHC^{14}O_3$, 0.2 ml enzyme, 6 micromoles phosphoenolpyruvate, total volume 1.5 ml. Incubated 60 minutes at 37° C. Reaction stopped with 0.1 ml 1 *N* HCl and the unreacted $C^{14}O_2$ removed by bubbling N_2 through the mixture. An 0.2-ml aliquot of the reaction mixture was counted with an end-window Geiger tube.

fixation of CO_2 in tobacco leaves. The isolation of labeled malate from young tobacco leaves exposed to $C^{14}O_2$ in the dark by Stutz and Burris (17) gives further evidence for the presence of a carboxylation reaction. Mazelis and Vennesland (10) have shown that the enzymes phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase are widely distributed in plant tissues. These results suggest the general occurrence of a mechanism for the dark fixation of CO_2 in leaves of higher plants similar to those we have proposed for both *Bryophyllum calycinum* (12) and *Nicotiana tabacum*. Vickery and Pucher (21) have proposed the presence of a similar reaction to account for the accumulation of citrate in tobacco leaves cultered in bicarbonate solutions in the dark. Incorporation of bicarbonate into citrate could take place via an initial carboxylation reaction to form a 4-carbon dicarboxylic acid which subsequently condenses with acetate.

The suggestion has been made by Bradbeer et al (3) that the initial carboxylation in the dark CO_2 fixation is on ribulose diphosphate. This unstable β-keto intermediate is immediately cleaved to phosphoglycerate and is subsequently metabolized to the 3-carbon acceptor phosphoenolpyruvate. This compound is then carboxylated to form the 4-carbon dicarboxylic acid. We have not been able to identify labeled phosphoglycerate in our short term experiments in either tobacco or Bryophyllum leaves exposed to $C^{14}O_2$ in the absence of light. However, after 5 minutes of dark fixation radioactivity can be detected in phosphoglycerate. It is possible that the age or the previous condition of the leaves could account for the differences in our observations.

Excised leaves of *Nicotiana tabacum* were exposed to $C^{14}O_2$ in the dark for periods as long as 5 hours. A typical radioautograph is presented in figure 3. The rates at which the compounds incorporate the radiocarbon from $C^{14}O_2$ are listed in table III. The radio-

TABLE III

PRODUCTS FROM THE DARK FIXATION OF $C^{14}O_2$ BY N. TABACUM LEAVES

COMPOUNDS	1 MINUTE	5 MINUTES	15 MINUTES	30 MINUTES
Malate	47.1	46.4	55.2	59.8
Citrate	12.1	11.6	3.6	5.3
Isocitrate	2.3	1.5	1.8	2.6
Succinate	1.9	4.7	5.8	4.5
Fumarate	0.3	1.0	1.1	1.1
Aspartate	23.9	17.4	13.2	8.0
Alanine	2.3	4.3	10.0	13.5
Glutamate	3.2	1.2	3.4	1.5
Serine	2.4	0.8	0.8	0.4
Glycine	0.6	0.2	0.4	0.2
Glutamine		Trace	1.1	1.3
Histidine			Trace	Trace
Proline			Trace	Trace
Threonine			Trace	Trace
Hydroxy-proline			Trace	Trace
Arginine			Trace	Trace

Concentrated alcoholic extracts of tobacco leaves exposed to $C^{14}O_2$ for the indicated intervals were separated by 2-dimensional paper chromatography as described. Following location and identification of radioactive compounds by radioautography the activity in each compound was measured with an end-window Geiger tube.

Activities are expressed as percent of total activity counted on the chromatogram. Samples were counted within a statistical error of 5 %. Those compounds indicated as "Trace" had less than 0.1 % of the total activity.

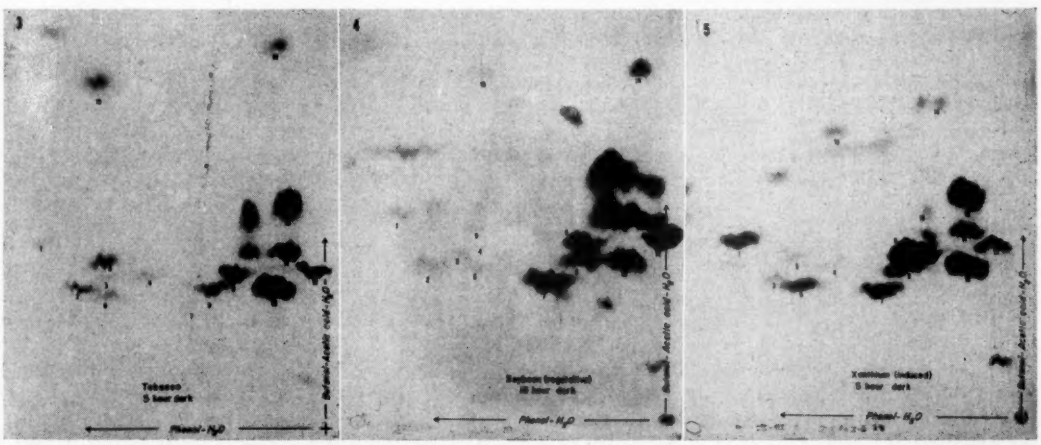


FIG. 3 Radioautogram of ethanol extract from tobacco leaf after 5 hours of dark $C^{14}O_2$ fixation. Compounds which have been identified are: 1. proline, 2. arginine, 3. hydroxy-proline, 4. threonine, 6. glutamine, 7. asparagine, 8. serine-glycine, 9. glutamate, 10. aspartate, 11. unidentified ester of malic acid, 12. *iso*citrate-citrate, 13. malate, 14. fumarate, 15. succinate, 16. glycerate.

FIG. 4. Radioautogram of extracts from soybean showing the absence of carbohydrates and phosphoralated derivatives. Compounds identified are numbered as in figure 3.

FIG. 5. Radioautogram of extracts from xanthium showing the absence of carbohydrates and phosphoralated derivatives. Compounds identified are numbered as in figure 3.

activities are expressed as percent of total activity counted on the paper at given time intervals.

In addition to the listed compounds, the labile α-keto acids, pyruvate, oxaloacetate and α-ketoglutarate were also isolated and identified as their 2,4-dinitrophenylhydrazones. There was no detectable activity present in glyoxylate. We have been unable to detect any radioactivity associated with the carbohydrate or phosphorylated carbohydrate fractions in tobacco leaves exposed to $C^{14}O_2$ in the dark for 5 hours. Sen and Leopold (14) report a rather significant carbohydrate fraction, 7 to 77 %, of the $C^{14}O_2$ fixed in the dark after 16 hours by Biloxi soybean, cockelbur, and Wintex barley. Our attempts to confirm the results of Sen and Leopold by exposing detached leaves of the above plants to $C^{14}O_2$ for 16 hours have thus far been unsuccessful. Radioautographs of the extracts from soybean, cockelbur are shown in figure 4. It would appear in leaves and other organs of higher plants that light is necessary for the generation of the reducing power needed to reverse the glycolytic pathway. However, germinating castor beans (16) can incorporate $C^{14}O_2$ into carbohydrates in the absence of light under conditions where fat is transformed into sugar.

The presence of an active Krebs cycle in photosynthesizing tissues has been an elusive problem for a long time. Recently, Smillie (15) has resolved this problem by preparing active cytoplasmic particles from green pea leaves, thus clearly establishing for the first time the operation of an active Krebs cycle in green leaves. We have been able to identify and isolate essentially all of the intermediates of the Krebs cycle with the exception of cis-aconitate. Although we have not demonstrated directly the enzymatic systems present in the cycle, the incorporation of $C^{14}O_2$ into cycle acids and related amino acids suggests that the cycle is operative in tobacco leaves.

The results obtained by Vickery et al (20) in their studies on the metabolism of excised tobacco leaves in various culture media strongly support the operation of an active Krebs cycle. Malate, succinate, and fumarate were shown to be equally effective as precursors in the formation of citrate. With succinate as a substrate significant amounts of malate accumulated. A decrease in malate concentration was observed when fumarate was used. Vickery et al (20) feel that these observations are not compatible with the operation of the Krebs cycle since fumarate should be a mandatory intermediate in the conversion of succinate to malate. It is possible to formulate another hypothesis consistent with the operation of the Krebs cycle to explain the above results. If one considers that malate accumulation is controlled by the ability of the plant cell to transport this acid into a metabolically inactive area of the cell such as the vacuole, it is possible that the addition of fumarate in high concentrations could inhibit this transport mechanism resulting in a decreased concentration of malate. Preliminary experiments in our laboratory indicate that differential vacuolar transport systems are operative. This inhibition of malate accumulation by fumarate would not in any way affect the metabolic pathway leading to the formation of citrate. Vickery et al (20) report that samples cultures in succinate did not accumulate any detectable fumarate. Therefore, succinate is converted to citrate and the accumulation of malate occurs in the absence of the inhibitory effects of abnormally high concentration of fumarate.

Glycolate did not incorporate radiocarbon in the dark when leaves were exposed to $C^{14}O_2$ for as long as 5 hours in the absence of light. It was reported earlier by Benson and Calvin (2) that in Chlorella, Scenedesmus and barley leaves labeled glycolate was found only during photosynthesis. Clagett, Tolbert and Burris (4) demonstrated that glyoxylate is formed by the direct oxidation of glycolate in plants. Kenton and Mann (8) found that glyoxylate can be oxidized to oxalate in tobacco leaves. Our inability to find labeled glycolate, glyoxylate or oxalate in excised leaves of tobacco exposed to $C^{14}O_2$ in the dark would appear to be in complete agreement with the known biosynthetic pathways of these compounds in leaves of higher plants. Although oxalate is one of the major acid components of tobacco leaves (19), we have been unable to demonstrate the incorporation of radiocarbon from $C^{14}O_2$ into this acid after 5 hours in the absence of light.

Serine incorporated C^{14} prior to glycine in tobacco leaves, as has been observed with Bryophyllum (13). Serine has been demonstrated to be precursor to glycine in animal tissues (6). Whether serine is synthesized in higher plants by a phosphorylated or non-phosphorylated pathway cannot be determined at the present time, since we have found both glycerate and phosphoglycerate labeled in the plant extracts.

Tolbert and Cohan (18) have shown that the major products formed from glycolate in barley and wheat leaves are glycine, serine and an unknown compound. There is a direct conversion of the glycolate to glycine and to the carboxyl and α-carbon of serine. The β-carbon of serine is formed from the α-carbon of glycolate. This evidence clearly establishes a direct biosynthetic pathway for the synthesis of glycine from glycolate. Therefore, it appears that there are two separate pathways present in leaves of higher plants for the biosynthesis of serine and glycine. It is difficult to assess the relative importance of the two pathways. Certainly in the dark, where CO_2 fixation in glycolate is not observed, radioactive serine arises from a 3-carbon precursor.

The striking qualitative similarity of the metabolic products involved with the dark fixation of CO_2 in excised leaves of *Nicotiana tabacum* and *Bryophyllum calycinum* are apparent from these studies. Although the rate of the initial CO_2 fixation in the absence of light is comparable and the mechanism of the initial carboxylation reaction appears to be identical, the succulents appear to have a unique mechanism for the storage of the synthesized organic acids which might account for the accumulation of a large quantity of the fixed carbon dioxide. We are not able to account for the difference at this time.

Summary

1. Excised leaves of *N. tabacum* have the ability to incorporate $C^{14}O_2$ in the dark into several organic and amino acids. These compounds have been identified by paper chromatography and radioautography.

2. The initial rate of dark CO_2 fixation in tobacco is more rapid than that of the succulent *B. calycinum*. However, the total amount of CO_2 fixed by tobacco after extended periods is much less.

3. The initial fixation of $C^{14}O_2$ appears to be mediated by the enzyme phosphoenolpyruvate carboxylase. The presence of this enzyme in tobacco leaves has been established.

4. The pattern of organic and amino acids which incorporate $C^{14}O_2$ suggest the operation of the Krebs cycle and concomitant transaminations. Serine seems to be the precursor to glycine in the dark.

5. No carbohydrates or phosphorylated sugars are labeled after extended periods of dark incorporation of $C^{14}O_2$. These findings suggest that light is needed to furnish the reducing power to reverse glycolytic processes.

We wish to acknowledge the valuable suggestions and assistance of Dr. John L. Webb in the preparation of this manuscript.

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INTERNAL DISTRIBUTION OF COENZYME Q IN HIGHER PLANTS [1, 2]

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Coenzyme Q is a lipid soluble quinone which has been shown to be an integral part of the electron transport system of beef heart mitochondria (3, 6, 10). The coenzyme Q isolated from beef heart mitochondria, previously referred to as Q_{275}, has been shown to be 2,3-dimethoxy-5-methyl benzoquinone containing 10 monounsaturated isoprenoid units substituted in the 6 position (5, 13, 17). This compound with 10 isoprenoid units in the side chain has been specifically named coenzyme Q_{10}. Coenzyme Q_{10} has also been found in all other mammalian tissues examined. It has been isolated from pig heart by Fahmy et al (7). Material with similar absorption spectra but with a lower melting point and higher extinction coefficient has also been isolated from animal tissues by Morton et al (8, 16). Lester and Crane (12) have isolated 4 different forms of coenzyme Q (Q_{275}) from microbial sources. Each of these forms has the same characteristic absorption spectrum (peak at 275 mμ) as coenzyme Q_{10} from heart, but a lower melting point and higher extinction coefficient which are consequences of the fact that the side chains of these forms have fewer isoprenoid units than coenzyme Q_{10}. These compounds have been called coenzyme Q_9, coenzyme Q_8, coenzyme Q_7, and coenzyme Q_6 (13).

All tissues of higher plants which have been examined contain coenzyme Q_{10}. During the examination of leaf tissue, however, another lipid-soluble quinone was found to be present in even larger amounts than coenzyme Q_{10}. This compound is characterized by an absorption peak at 255 mμ and it has been called Q_{254} (4). (In less purified preparations the maximum is at 254 mμ.) Under the conditions used for enzymatic assay of Q_{10}, Q_{254} shows coenzyme Q activity which is defined as: 1) the ability to restore succinoxidase activity in beef heart mitochondria after isooctane extraction and 2) the ability to undergo reduction in the presence of mitochondria, succinate, and cyanide and 3) the ability of the hydroquinone to undergo oxidation in the presence of mitochondria and oxygen (3, 4). The localization of both Q_{10} and Q_{254} in the particulate fractions obtained by differential centrifugation of a spinach leaf homogenate will be described in this communication as well as other evidence that Q_{254} is concentrated in the chloroplasts whereas Q_{10} is concentrated in mitochondria.

METHODS

Spinach leaves were obtained from a local market. The main veins were removed. Three hundred eighty grams of leaves (equivalent to 42 g dry weight) were homogenized in 4 equal batches—each batch containing 300 ml of 0.5 M sucrose, 30 ml 0.5 M tris acetate, pH 7.5, and 95 g of leaves. Homogenization was carried out in a Waring blendor for 1 minute at half speed. The homogenate was strained through cheesecloth. The filtered homogenate was then repeatedly centrifuged at progressively higher speeds to separate the particles. The conditions for the centrifugations were as follows: 1) 5 min at 100 × G, 2) 10 min at 200 × G, 3) 10 min at 600 × G, 4) 20 min at 3,000 × G in the 8-place angle head of the International PRI, and 5) 30 min at 78,000 × G in the no. 30 head of the Spinco model L centrifuge. The final supernatant (fraction 6) was clear with a deep yellow color. The precipitate obtained after each centrifugation was taken up in 0.5 M sucrose and homogenized in a Potter-Elvehjem homogenizer. The dry weight of each of the fractions, as well as recovery of material, is listed in table I.

TABLE I

DISTRIBUTION OF DRY WEIGHT, COENZYME Q, CHLOROPHYLL, AND SUCCINIC DEHYDROGENASE IN SPINACH LEAF FRACTIONS*

FRACTION	DRY WT TOTAL MG	Q_{254} TOTAL MG	CHLOROPHYLL TOTAL AS 665 mμ	COENZYME Q TOTAL MG	SUCCINIC DEHYDROGENASE* TOTAL UNITS
Homogenate	30,000	4.60	5,270	0.70	...
1	760	0.17	258	0.03	...
2	1,400	0.95	1,160	0.03	0.50
3	2,180	2.65	2,710	0.11	2.66
4	1,050	0.48	594	0.22	2.84
5	1,640	0.35	460	0.15	1.64
Supernatant	25,300	0.00	0	0.00	...
Recovery	32,330	4.60	5,190	0.54	
Original leaf	42,000	4 62	...	1.51	...

* Succinic dehydrogenase expressed as μM succinate oxidized × 100 per min by the total fraction.

Coenzyme Q_{10} was extracted as described for beef heart mitochondria (6) by shaking the acidified suspension (KH_2PO_4) with isooctane. The isooctane was evaporated and the residue taken up in ethanol. Coenzyme Q_{10} in the ethanolic solution was determined by observing the change at 275 mμ which occurred upon addition of solid potassium borohydride to the solution. Q_{254} was found in the same isooctane extract

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as coenzyme Q_{10} and was determined by measuring the change at 254 mμ induced by addition of borohydride. (When coenzyme Q_{10} was also present a correction must be made for the change in absorbance at 254 mμ during reduction of coenzyme Q_{10}. This change represents one half the change at 275 mμ.)

The extinction coefficient for coenzyme Q_{10} at 275 mμ is taken as $E^{1\%}_{1cm} = 165$, with $\triangle E^{1\%}_{1cm}$ at 275 mμ (oxidized-reduced) equal to 142. The use of this value is justified by the fact that the coenzyme Q from spinach shows the same chromatographic behavior as coenzyme Q_{10} from beef heart and alfalfa (2, 15). The extinction coefficient for Q_{254} at 254.0 mμ is taken as $E^{1\%}_{1cm} = 210$ and the $\triangle E^{1\%}_{1cm}$ (oxidized-reduced) = 198. These values are based on measurements with Q_{254} isolated from alfalfa (2) which shows the same chromatographic behavior as a purified sample of Q_{254} from spinach leaves.

No significant amount of vitamin K appears in the extracts obtained by the procedure described. Vitamin K can be determined by a characteristic increase in absorption at 240 mμ when reduced with borohydride whereas the coenzyme Q compounds show a slight decrease in this region. If extracts are obtained which contain vitamin K which will interfere in the assay of coenzyme Q compounds it can be separated from the coenzyme Q_{10} and Q_{254} by chromatography on Decalso as described below for extracts from corn. The vitamin K appears in the eluate from the column before the coenzyme Q compounds.

Chlorophyll was determined by measuring the absorbance at 665 mμ of a methanolic extract obtained from an aliquot of each fraction. To obtain the methanol extract 0.5 ml of the sample was extracted with 5 ml boiling methanol in presence of 1 ml 0.1 M TRIS [tris (hydroxymethyl) aminomethane] chloride pH 8.0. Succinic dehydrogenase was determined by the succinic ferricyanide assay (9).

Results

1. Distribution of Coenzyme Q and Q_{254} in Particulate Fractions from Spinach Leaves: The homogenate which is obtained after filtering the blendorized leaves through cheesecloth is deep green in color, while the fibrous material remaining in the cheesecloth has a pale green color. The homogenate contains most of the Q_{254} of the leaf, but only about one half of the coenzyme Q_{10} contained in the leaf is found here.

Both Q_{254} and Q_{10} are contained exclusively in the particulate fractions obtained by centrifugation. Q_{254} is concentrated in the dark green particles which sediment at low centrifugal force, and its distribution in the particulate fractions is closely correlated with the chlorophyll content. On a dry weight basis Q_{254} is concentrated about 10-fold in the fraction with the highest chlorophyll content over the concentration found in leaves. Q_{10}, on the other hand, is concentrated in the pale green particles which sediment at higher centrifugal force, and its distribution is correlated quite well with the distribution of succinic dehydrogenase activity in the particles (cf. fig 1). The Q_{10} content in the particles with the highest succinic dehydrogenase activity is about 5 times that of the whole leaf.

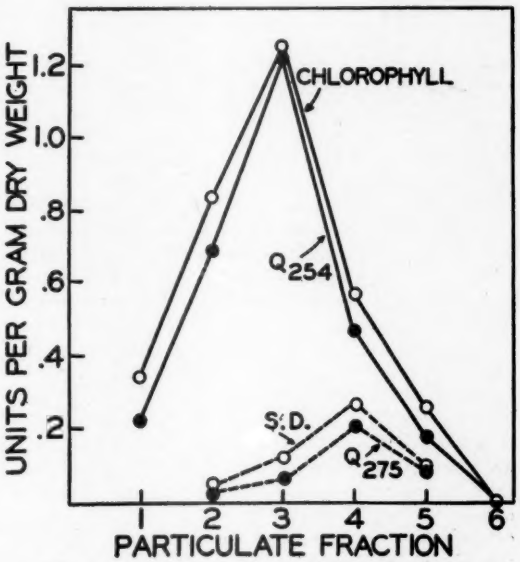

Fig 1. Concentration of coenzyme Q (Q_{275}) and Q_{254} in relation to chlorophyll and succinic dehydrogenase content of spinach leaf particles. Concentration of chlorophyll, ○; Q_{254}, ●; coenzyme Q (Q_{275}) --○--, and succinic ferricyanide activity (S.D.) --○--, are in the following units: chlorophyll total absorbance at 665 mμ/mg dry weight, succinic dehydrogenase μM of succinate oxidized × 100/min × mg dry weight, coenzyme Q and Q_{254} mg/g dry weight.

2. Distribution of Coenzyme Q_{10} and Q_{254} in the Variegated Leaves of Pandanus: The correlation between the distribution of Q_{254} and chlorophyll was further investigated by determining the distribution of these compounds in the white and green portions of vertically striped leaves of *Pandanus vetchii*. Two leaves were selected in which one half of each leaf was white and the other half was green. The white and green portions of the leaves were separated into 2 separate samples. The leaf material was finely cut up and extracted by refluxing with 20 ml of 50 % ethanol 50 % isooctane (with 0.3 ml 1 M phosphate buffer pH 7.4 added). The material was protected from light during this operation. The extracts were then assayed for Q_{10} and Q_{254} by change of absorbance at 275 and 254 mμ following addition of borohydride. Coenzyme Q_{10} and Q_{254} were both found in the extracts from the green and white portions of the leaves. There was, however, 10 times as much Q_{254} in the green portion of the leaves as in the white;

TABLE II

COENZYME Q_{10} AND Q_{254} CONTENT OF WHITE AND GREEN PORTIONS OF PANDANUS LEAVES

LEAF SECTION	AMOUNT EXTRACTED G FRESH WEIGHT	Q_{10} MG/G*	Q_{254} MG/G*	CHLOROPHYLL AS 665 $M\mu$/G*
Green	4.04	0.0057	0.0173	38.6
White	3.77	0.0018	0.0017	1.0

* Values based on fresh weight of tissue.

while there was only 3 times as much coenzyme Q_{10} in the green fraction as in the white (cf. table II).

3. DISTRIBUTION OF COENZYME Q AND Q_{254} IN CORN SEEDLINGS: The distribution of the 2 quinones in the roots and leaves of corn seedlings has also been determined. Corn seeds (*Zea maize* var. Golden Glow) were soaked in water and germinated in sand. Seven days after planting the plants were harvested, and the roots and shoots were detached from the seeds. Samples of the roots and shoots were dried for 48 hours at 100° C. After the dried tissue was ground in a mortar to a fine powder, 1-g portions of each tissue were placed in glass-stoppered test tubes and extracted by vigorous shaking with three 10-ml portions of isooctane containing 5 % ethanol. The isooctane extracts were then combined and the solvent evaporated. The oily residue was taken up in isooctane and placed on a small column of Decalso (10 cm × 1.5 cm). The column was eluted successively with 100 ml of isooctane, 200 ml of 5 % ether in isooctane, 100 ml of 10 % ether in isooctane, 100 ml of 10 % ethanol. The bulk of the coenzyme Q and Q_{254} is contained in the 5 % ether eluate, although occasionally some quinone is found in the 10 % ether eluate. Each of the eluates was evaporated and the residue taken up in a known volume of ethanol. Coenzyme Q and Q_{254} were estimated in the ethanol solution. All values for coenzyme Q in these extracts are expressed as coenzyme Q_{10}.

Consistent with the previous results which show a tendency for Q_{254} to be concentrated in the green portions of plants, there is more Q_{254} than coenzyme Q in the corn shoots and less Q_{254} than coenzyme Q in the roots (cf. table III). It is also noteworthy that the young shoots of corn contain a one third higher concentration of coenzyme Q than spinach leaves, whereas the concentration of Q_{254} is less than in spinach leaves.

TABLE III

COENZYME Q AND Q_{254} IN ROOTS AND SHOOTS OF CORN SEEDLINGS

	COENZYME Q*	Q_{254}*
Roots	0.034	0.018
Shoots	0 059	0.082

* Quinone concentration expressed as mg of the quinone per gram dry weight of tissue.

DISCUSSION

The association of coenzyme Q_{10} with the succinic dehydrogenase contained in spinach leaf particles is consistent with other observations that coenzyme Q_{10} is associated with mitochondria. In this laboratory we have found that this quinone is concentrated in mitochondria from beef heart and beef liver as well as in pigeon breast muscle cyclophorase. Coenzyme Q_9 in *Azotobacter vinelandii* has been found to be concentrated in the electron transport particle from that organism (14). Hemming, Pennock, and Morton have also reported the presence of a coenzyme Q type material in rat liver mitochondria (11).

The broad distribution of the 5 known forms of coenzyme Q in aerobic tissues (14) is also consistent with the view that these compounds are necessary for respiratory electron transport which occurs in mitochondria or equivalent structures. Forms of coenzyme Q have been found in all vertebrate tissues and in all higher plants examined as well as in many aerobic bacteria, Baker's yeast, certain invertebrates, and in members of the green algae and red algae. On the other hand, it is not found in yeast grown under anaerobic conditions nor in the anaerobic bacteria *Clostridium perfringens*.

There are still some exceptions to the pattern of association of coenzyme Q with aerobic tissues. It has not been found as yet in certain invertebrates, nor in members of the brown algae, basidiomycetes or certain aerobic bacteria. The possibility that some other form of coenzyme Q which is not detected by our assay methods exists in these tissues is not excluded. It is also possible that other quinones fulfill the function of coenzyme Q. Thus polyporic acid in the basidiomycetes and vitamin K in Mycobacteria (1) could well be considered in relation to respiratory function.

In contrast to the general distribution of coenzyme Q, Q_{254} has been found primarily in tissues capable of photosynthesis, and it has been found in high concentration in all chlorophyll-containing tissues examined. These include members of the higher plants as well as green, brown and red algae. It has not been found in the photosynthetic bacteria *Rhodospirillum rubrum* or in Chromatium (strain D), both of which contain, however, large amounts of coenzyme Q.

General distribution of Q_{254} in chloroplasts reported here is consistent with the view that Q_{254} is a component of chloroplasts and that it is not involved in respiratory electron transport, but that its function should be sought in photosynthetic electron transport.

There remains some discrepancy in the above interpretation in that small amounts of Q_{254} are found in such tissues as cauliflower buds (4), corn roots, and the white portions of variegated plants. It is possible that these tissues, which could be considered potentially capable of photosynthesis, contain proplastids which contain Q_{254}. Further work to define the particulate components of the cells of these tissues is needed to provide a final answer to this problem.

SUMMARY

The distribution of two quinones with coenzyme Q activity which are broadly distributed in higher plants has been determined. Particulate fractions from spinach leaves, the white and green portions of Pandanus leaves, and the roots and shoots of corn seedlings have been examined with respect to the amount of coenzyme Q and Q_{254} present. Coenzyme Q is found in both white and green tissue and appears to be concentrated in mitochondria, whereas Q_{254} is concentrated in chloroplasts and chloroplast-containing tissue. It is proposed that coenzyme Q is involved in mitochondrial electron transport and that Q_{254} may be involved in photosynthetic electron transport.

I wish to thank Dr. D. E. Green for his encouragement of this investigation, Dr. Robert L. Lester for paper chromatography of the quinones, and Mrs. Wanda Fechner for her excellent technical assistance. *Pandanus vechii* was kindly supplied by Dr. R. H. Roberts of the Department of Horticulture, University of Wisconsin.

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STUDIES OF THE REST PERIOD. I. GROWTH, TRANSLOCATION, AND RESPIRATORY CHANGES IN THE EMBRYONIC ORGANS OF THE AFTER-RIPENING CHERRY SEED [1,2]

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Rest or dormant periods are common in the plant kingdom, and have been widely studied in such organs as buds, seeds, tubers, and corms. The seed provides particularly favorable material for investigating the rest period since it may, to a considerable degree, be considered a closed system, complete with its store of organic materials, minerals, and growth factors, and depending on the external environment only for water, oxygen, and favorable temperature and light conditions. The dry seed contains an embryonic plant in an arrested condition; germination may be considered to be the resumption of growth. Toole et al (18) and Evenari (4) have pointed out that germination requires the coupling of respiratory energy to growth, but that little is known concerning the actual mechanisms of germination.

While the seed may be considered to be a closed

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system, it cannot be treated as a homogeneous system since it is structurally complex with physiological specialization closely associated with this structural complexity. Generally speaking, the potentiality for active synthesis and growth is restricted to the embryonic axis, while the cotyledons and/or endosperm are primarily concerned with the storage and subsequent mobilization of reserve materials. Since a seed is heterogeneous and complex, investigations of the germination process must treat the seed in its complexity and study the physiology of the individual organs and their interrelations. Such an approach was suggested by Sherman (15) but has received relatively little experimental treatment.

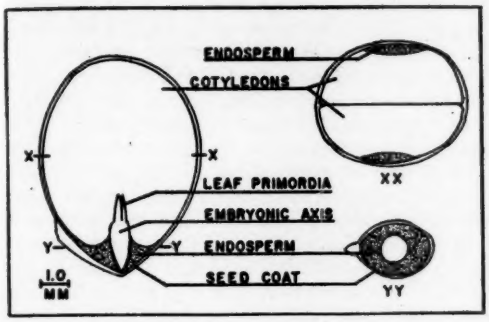
Most dry seeds contain all essential elements for germination except water. However, in many seeds the simple uptake of water will not permit germination because the growth of the embryo is blocked by the presence of a resting or dormant condition (see (2) and (13) for discussion of terminology). Crocker (3) described 6 classes of blocked growth which are obviously associated with blocks at different stages of germination and in different parts of the seed. It is clear in Crocker's classification that mechanical restriction of transport or expansion is relatively simple compared with "embryo dormancy" and "secondary dormancy." These latter, while classified by Crocker as distinct types, probably are identical (1, 6, 17, 20) and are closely similar to, if not identical with, the rest period of buds of trees (1, 6). We will refer to the result of any block to growth which resides within the embryo as the rest period.

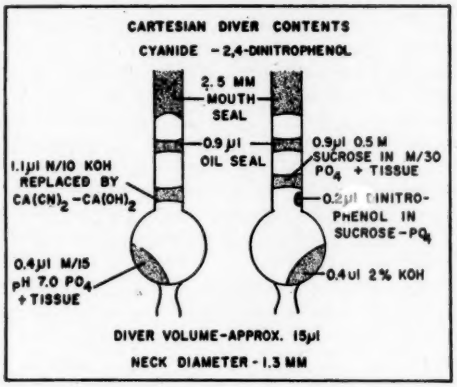
The rest period is frequently studied because of its importance in agriculture and forestry. However, rest is also of great theoretical importance because it results from a block to growth within the growing cells; understanding the nature of this block could contribute directly to the understanding of the factors which control cellular growth and development. However, it is much easier to define the rest period in writing than it is to measure it in the laboratory. For example, germination is generally measured as the emergence of the primary root from the seed coat. However the question arises as to whether growth by cell division or enlargement prior to rupture of the seed coat is part of germination. To avoid this difficulty Toole et al (18) defined germination as "the resumption of growth" and Evenari (4) considers germination to be those processes preceding the resumption of growth. The rest period may be defined as a blocking of germination, but we must recognize that this is not an adequate working definition for at least 2 reasons: 1) The block to growth may not be complete, thus some growth may continue during the rest period as in the embryos described in this paper; and 2) the rest period can only be defined in terms of failure to grow. In the laboratory, it is normal practice to remove the block to growth by after-ripening at low temperatures and then to test for growth by placing the material at higher temperatures. There is, however, ample evidence (1) that these higher temperatures themselves can reverse the effects of low temperature and tend to re-establish the rest period; according to Crocker's terminology (3), secondary dormancy is established. Thus it is essential to have a measure of the actual growth potential of the system which at least minimizes this reversing effect. It is for this reason that recent workers such as Abbott (1) and Visser (20) use a more comprehensive measure of "germination capacity" rather than germination, and a similar procedure is followed in the work presented in this paper.

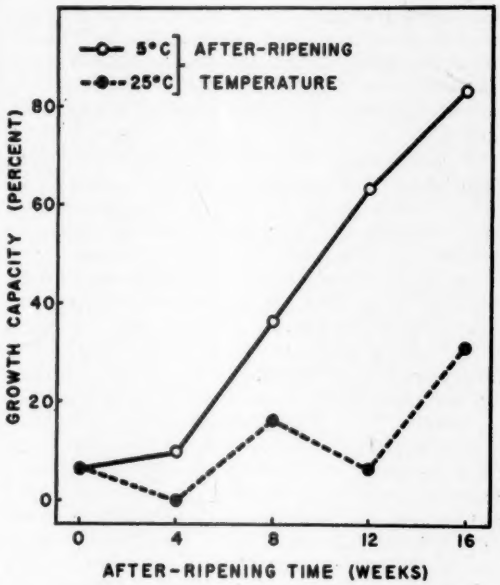
In the experiments reported in this paper the cherry seed was chosen as experimental material since it contains an embryonic axis with a well developed root and shoot, has a definite rest period (8, 15, 19), and is readily available. In early experiments, we found that low-temperature after-ripening increases the respiration rate of cherry seeds. We then investigated the relative effect of low temperature on the various seed organs and found that the respiration of the parts of the embryonic axis increases much more than the whole seed. Immediately following this respiratory stimulation is growth by cell division and probably also cell enlargement, together with changes in dry weight due to translocation and utilization of nutrients. Because these changes tend to complicate the interpretation of the respiratory data by changing the basis for expression (respiration per unit dry weight, cell, or organ) and because few data of this type are available in the literature (18), we have included a rather detailed study of this type of change. We have also analyzed the respiratory increases, making use of 2,4-dinitrophenol (DNP) to permit determination both of respiratory rate and respiratory capacity as measured by the maximum respiration rate obtained in the presence of DNP. The results of these experiments suggest that the initial effect of low-temperature after-ripening is to increase the efficiency with which respiratory enzyme systems operate, thus providing a greater supply of available energy to the embryo and triggering a complex of reactions leading eventually to active growth.

Materials and Methods

Plant Material: Seeds of the sour cherry, *Prunus cerasus* L. var. Montmorency, from the 1957 crop of the University of Delaware farm were used for the bulk of the work reported here, while seeds from the 1954, 1955, and 1956 crops were used for preliminary experiments. No difference was noted between these several crops. The fruits were pitted immediately after harvest and the pits were thoroughly washed and allowed to air-dry in a single layer at room temperature. They were stored at room temperature until needed, at which time they were cracked. The undamaged seeds were sterilized by immersing for 10 minutes in a filtered suspension of 5% Pittchlor (Columbia-Southern Chemical Company) in water. After-ripening was carried out on




CARTESIAN DIVER CONTENTS
CYANIDE - 2,4-DINITROPHENOL
2.5 MM MOUTH SEAL
0.9 µl OIL SEAL
1.1µl N/10 KOH REPLACED BY CA(CN)2 - CA(OH)2
0.9µl 0.5 M SUCROSE IN M/30 PO4 + TISSUE
0.2µl DINITRO-PHENOL IN SUCROSE - PO4
0.4µl M/15 pH 7.0 PO4 + TISSUE
0.4 ul 2% KOH
DIVER VOLUME-APPROX. 15µl
NECK DIAMETER - 1.3 MM



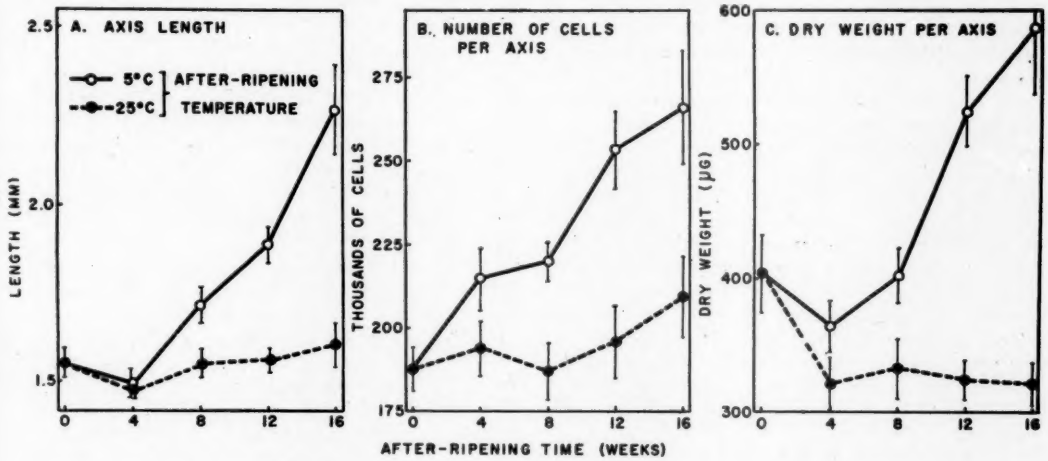


FIG. 1 (*upper left*). A diagram of the structure of the cherry seed.

FIG. 2. (*center left*). Cartesian diver contents in experiments with 2,4-dinitrophenol and cyanide.

FIG. 3 (*upper right*). Growth capacity of cherry embryos as a function of time and after-ripening temperature. Growth capacity is based on relative growth rates and stage of development reached by excised embryos during a 2-week period at 25° C.

FIG. 4 (*bottom*). The effect of after-ripening time and temperature on growth and dry weight in the embryonic axis of cherry seeds. The vertical lines represent ± the standard error of the mean.

sterile moist sand, with water added as needed to keep the seeds moist but not submerged. For most purposes, approximately 40 seeds were after-ripened in a 125 ml Erlenmeyer flask. However, there occasionally seems to be an interaction between seeds in such a flask when 1 seed germinates and stimulates germination of several surrounding seeds. To avoid this complication, most of the data used in this paper were obtained from seeds after-ripened individually on sand in 13 × 120 mm test tubes. After-ripening was carried out in the dark at 5° C and at 25° C; any contaminated or structurally abnormal seeds were discarded.

Figure 1 shows the structure of the cherry seed. Germination in this seed is epigeal; the embryonic leaf primordia become the 1st true leaves of the seedling. Dissection, carried out with the seed submerged in water and observed with a 10 × dissecting microscope, involved separating the cotyledons from the embryonic axis by diagonal cuts at the cotyledonary node. The leaf primorida were removed by cutting at the point of petiole attachment. Thus the portion of the embryo referred to as "embryonic axis" includes the root primordium, hypocotyl, and shoot apex.

Germination and growth capacity were measured as part of a study on growth rates according to the following procedure: 500 ml tall form beakers were lined with germination blotting paper, partially filled with washed sand moistened with water, covered with a Petri dish half, and sterilized. Seeds, 15 per beaker, were inserted between the blotting paper and glass in such a position that they could be observed and measured with a horizontal microscope. All tests were performed at 25° C in the dark except for brief periods of observation under room light. Germination was measured by placing whole seeds in the beakers; a seed was considered to have germinated if the root emerged from the seed coat within 2 weeks. However, in closely related seeds, Abbott (1) and Visser (20) have shown that growth in embryos excised from the seed coats is a better measure of germination ability than that described above for whole seeds. Since we were also interested in obtaining visual observations on changes in the leaf primordia during germination, seed coats were excised, 1 cotyledon was removed, and the embryonic axis with 1 cotyledon was placed in the beaker with the axis toward the glass for observation and measurement. As a measure of growth capacity, the seeds were divided into 4 classes according to the changes occurring within a 2 week period:

Class 1: Embryos not growing: Leaf primordia and axis with little or no elongation. Color of embryo mostly white with occasionally yellow areas.

Class 2: Slight growth: Leaf primordia enlarging and turning yellow. Some thickening and yellowing of the axis. Cotyledons white with occasional yellow spots.

Class 3: Weak growth: Leaf primordia enlarging and turning yellow. Considerable elongation in the axis with the yellowing of the root tip. Partial yellowing of the cotyledons.

Class 4: Active growth: Rapid elongation and yellowing of the leaf primordia and axis, together with yellowing of the cotyledons.

These classes were assigned the values 0, 1/3, 2/3, and 1 respectively. Then the number of embryos in each class multiplied by the class value, divided by the number of embryos tested (20 to 30), and expressed as percent, was taken as the growth capacity of the material. The results of this experiment are shown in figure 3.

Analytical Methods: If we examine a cherry seed, we find that the amount of experimental material available in the embryonic axis and leaf primordia is only a very small part of that in the whole seed. For example, an average non-after-ripened seed has a dry weight of 56.5 mg and contains an embryonic axis with a dry weight of 405 μg and 2 leaf primordia each with a dry weight of about 12 μg. Thus in comparing changes in leaf primordia with changes in whole seeds, we are dealing with materials differing in size over a 1000-fold range. Since only a limited number of seeds can be dissected within a practical period of time, it has been necessary to use a range of methods, from the "conventional" to the "ultramicro."

Several measurements were made to compare the size of the seed parts and to serve as a basis for expressing other data. Linear measurements on the leaf primordia and embryonic axis were made with an ocular micrometer in a 10 × dissecting microscope. Dry weights were obtained on these same organs using "fishpole" type quartz fiber balances (12). Several balances were used; the size of the balances was chosen to give dry weights of the organs accurate to at least 1 %. Dry weights of whole seeds were obtained with a Roller-Smith balance sensitive to 0.02 mg. Cell counts were obtained using the maceration technique described by Sunderland and Brown (16). We have also studied changes in total nitrogen and phosphorus; these data will be presented in another paper.

During the course of these investigations we attempted to obtain the volume of the embryonic leaf primordia and axis by the method of Lø vtrup (11). Briefly, this method involves measuring the reduced weight of an organism with the Cartesian diver balance (10) and the density from the level at which the organism comes to rest in a physiologically-inert density gradient. From these 2 measurements, the volume of the organism can be calculated. While the reduced weight measurement offered no problem, density measurement proved difficult. The axis and leaf primordia are relatively dense, 1.06 to 1.08, in comparison with the amoeba, density 1.02, for which the method was originally developed. The solutions available for these densities were so viscous that it was difficult to find a definite rest point in the gradient. We used a high molecular weight dextran preparation (kindly supplied by the Commercial Solvents

Corporation through Dr. F. Kavanagh), but even this preparation was quite viscous at these densities. Nevertheless, this method may in the future provide a convenient and accurate way of measuring the volume of meristematic plant tissues which have complex shapes.

Respiration, measured as oxygen uptake, was determined by 3 methods. In each case, the gas atmosphere was air and a temperature of 25° C was used. The rates were essentially constant for the period of 4 to 6 hours during which measurements were made. For whole seeds, standard Warburg techniques were used. Groups of 10 to 20 seeds were placed on moist filter paper in the bottom of a 20 ml vessel, and measurements were made without shaking. For the embryonic axis, the differential volumeter described by Grunbaum et al (7) was employed. The particular instruments used were made with capillaries of 0.8 to 1.0 μl/cm volume and approximately 1 ml flasks. The axes were placed, 5 in a flask, on moistened filter paper disks.

Respiration of leaf primordia was measured using standard Cartesian diver techniques ((9) and other papers from the Carlsberg Laboratory). Figure 2 illustrates the filling schedule for the various experiments. All solutions were prepared using water redistilled from an all-glass (Pyrex) still. The oxygen consumption per diver was kept at a minimum of about 10×10^{-3} μl/hr by using pairs of leaf primordia from non-after-ripened seeds and from seeds after-ripened at 25° C. With seeds after-ripened at 5° C a single primordium was placed in a diver. In most cases, this resulted in an oxygen uptake below 80×10^{-3} μl/hr/diver although a few ran as high as 140×10^{-3} μl/hr/diver. However, empirical measurements showed that even with these high rates, diffusion of oxygen to the primordium and absorption of carbon dioxide were not limiting.

In experiments with 2,4-dinitrophenol (DNP), the normal respiration rate was measured for a period of 2 to 3 hours before the seal containing the leaf primordium was mixed with a side drop containing DNP. The rate of oxygen uptake in the presence of DNP was allowed to stabilize for about 1 hour and the linear rate was then measured during the period 1 to 3 hours after mixing. Early experiments with DNP indicated that respiratory rates during these rather long experiments were stabilized somewhat if the primordia were suspended in 0.5 M sucrose in M/30 phosphate buffer pH 5.3. Other than this slight stabilizing effect, the sucrose used in this series of experiments seemed to have no influence on respiratory rates.

For cyanide inhibition measurements, the $Ca(CN)_2$–$Ca(OH)_2$ method of Robbie (14) was used, with the HCN concentrations calculated as for the Warburg apparatus. Two measurements were made for each primordium. First a normal rate was determined during a 2 to 3 hour period by absorbing the CO_2 in a seal of 0.1 *N* KOH. This seal was then replaced with the appropriate $Ca(CN)_2$–$Ca(OH)_2$ mixture and the linear rate of oxygen uptake was determined for a period of 2 to 3 hours after the initial exposure to HCN. Although in the Cartesian diver the $Ca(CN)_2$–$Ca(OH)_2$ mixture is not as effective in CO_2 absorption as KOH, probably because of the formation of a surface film of $Ca(CO_3)_2$, empirical measurements showed that the rate of CO_2 absorption did not become limiting.

The sampling procedure followed in these experiments involved analyses of non-after-ripened seeds and of seeds after-ripened for 4, 8, 12, and 16 weeks. The non-after-ripened seeds were sterilized and allowed to imbibe water for 2 to 5 days before analysis. At each of the subsequent time periods the measurements with living material were completed within a 3 to 5 day period. At each sampling time 1 group of 15 seeds from each treatment was removed from after-ripening conditions and was immediately excised and the respiration rate of 1 or both leaf primordia measured. One primordium was then used for cell counting and the other was dried and used for dry weight measurement and total nitrogen analysis. The embryonic axis was macerated for cell counting. Another group of 15 seeds was excised and the leaf primordia dried for total phosphate analysis while the embryonic axes, in groups of 5, were used for respiration measurements. These were then dried and used for dry weights and total nitrogen and phosphate analysis. Three groups of 10 seeds each were used for respiration measurements on whole seeds and were then excised for growth measurements. Finally, 20 to 30 seeds were used to measure the germination of intact seeds. It should be noted in the sampling procedure for leaf primordia that the primordia from any one seed are so closely matched that a "matched leaf" technique could have been employed.

Results

The data obtained show that after-ripening at 5° C leads to 5 % germination after 12 to 16 weeks: a high germination percentage is not achieved under these conditions until after 18 weeks. After-ripening at 25° C does not lead to normal germination. As shown in figure 3, excision of the embryo leads to much earlier growth, according to the classification used almost full capacity after 16 weeks at 5° C. Excision of embryos after-ripened at 25° C leads to a slight increase in growth capacity by 16 weeks.

One of the qualitative characters taken as a measure of growth is a change in the growing portions of the embryo from a white opaque to a yellow translucent appearance. This change is apparently due to 2 factors: 1) the synthesis of pigments and 2) the emptying of the reserve materials from the cells. The latter can be seen clearly in microscope sections of the material. This means that growth in these cells is accompanied by utilization of storage materials or their conversion into protoplasm and cell wall material in situ. For this reason, dry weight or total nitrogen may be poor bases for expressing other data. We have therefore investigated such other measures of growth as organ size and number of cells.

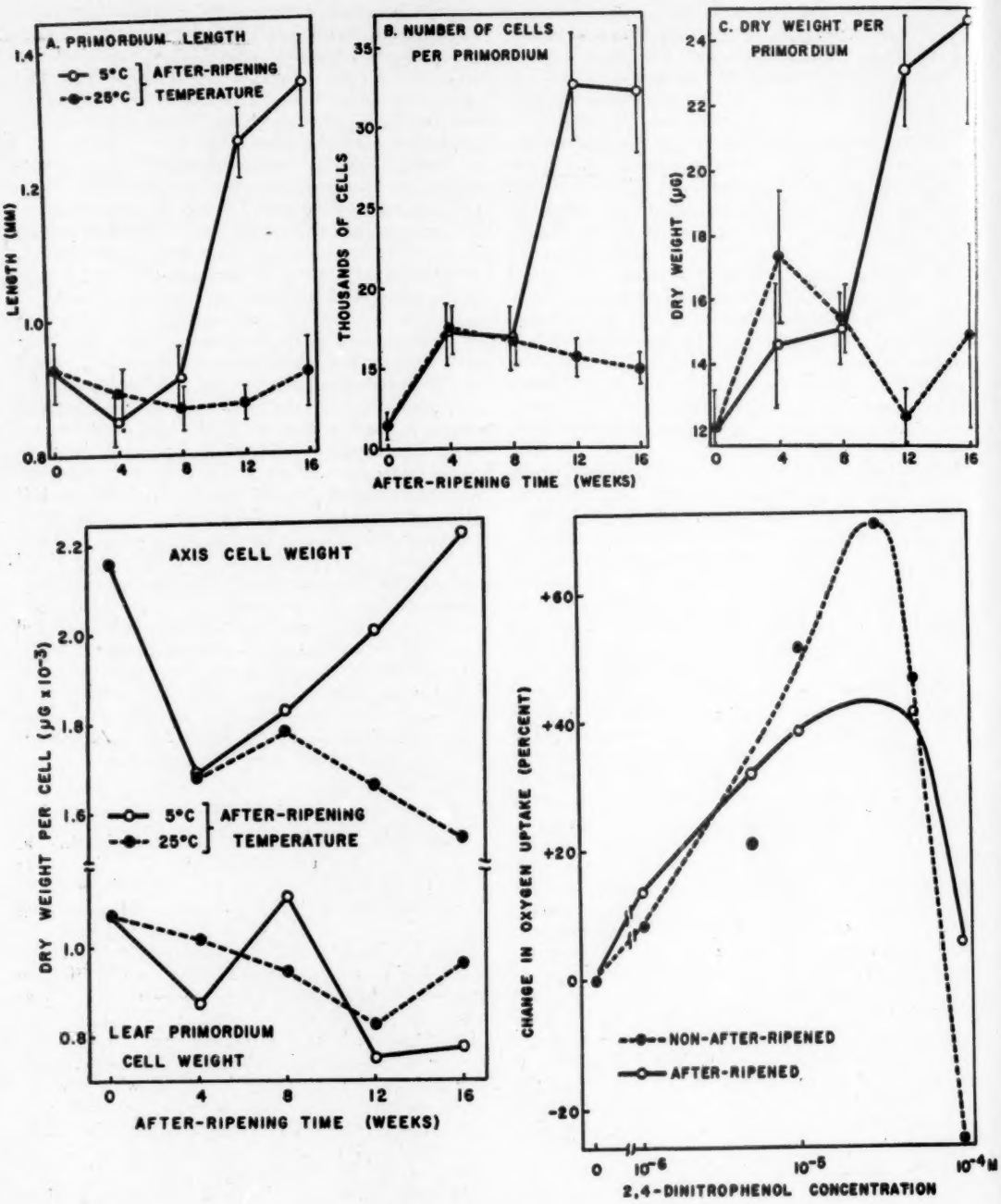


FIG. 5 (*top*). The effect of after-ripening time and temperature on growth and dry weight in the embryonic leaf primordia of cherry seeds. The vertical lines represent ± the standard error of the mean.

FIG. 6 (*lower left*). Changes in dry weight of the cells of the embryonic axis and leaf primordium as a function of after-ripening time and temperature.

FIG. 7 (*lower right*). The effect of 2,4-dinitrophenol concentration on the oxygen uptake of cherry embryonic leaf primordia.

In figures 4 and 5 are presented data on changes in the embryonic axis and leaf primordia during after-ripening at 5° C and at 25° C. A number of changes are obvious and fairly constant, although the exact times of these changes are not necessarily synchronized in the axis and leaf primordia. Specifically, at 5° C both the axis and leaf primordia show an increase in length roughly correlated with the increase in the growth capacity. This increase in length is due at least in part to the production of new cells. The increase in length and number of cells is also accompanied by an increase in the total dry weights of axis and leaf primordia (figures 4 C and 5 C). In the axis, the dry weight per cell tends to drop and then increase again (figure 6), while in the leaf primordia it tends to drop, although somewhat erratically because the rate of cell division is not constant. The difference in amount of variability between leaf primordia and axis in this respect is magnified by the fact that during the 1st 4 weeks of after-ripening the increase in cell number in the axis could be accounted for by a single division in 15 % of the cells, while in the leaf primordia it was the equivalent of a single division in 51 % of the cells. Thus cell division in the leaf primordia is proportionately much more active. It should be noted that the dry weight per cell in the leaf primordium is less than half that in the axis. This is at least partially the result of a difference in cell size as can be seen in microscope sections. Whether this also represents a difference in amount of stored material cannot now be determined.

In comparing embryos after-ripened at 5° C, and thus being prepared for active growth, with embryos after-ripened at 25° C and increasing little in growth capacity, a number of differences are obvious. According to most of the measurements made, increases with after-ripening time at 5° C are not paralleled by increases in embryos after-ripened at 25° C. However, if we examine the changes during the 1st 4 weeks of after-ripening, we find that these are actually quite similar at both 5° C and 25° C, the divergence appears in most cases only after 4 weeks.

It should be recognized that the growth shown in figures 4 and 5 can be seen only by means of the precise measurements used to obtain these data. On the other hand, the data used to estimate the growth

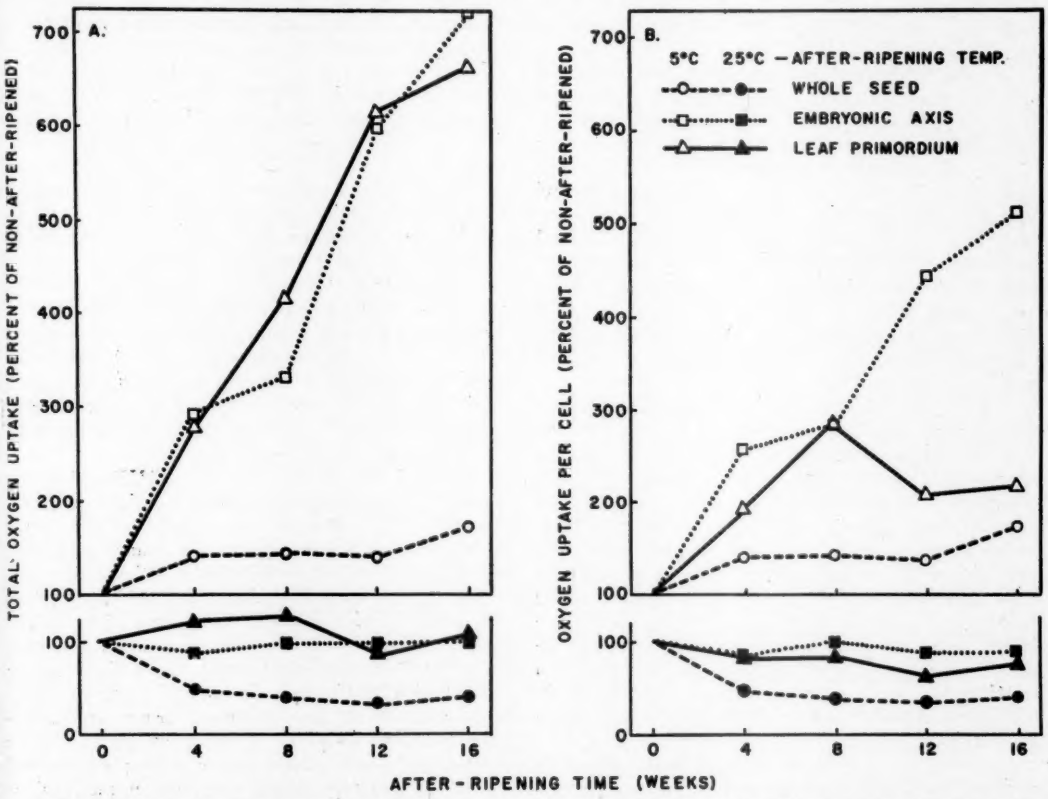


FIG. 8. Changes in rate of oxygen uptake of intact seeds, embryonic axes, and embryonic leaf primordia during after-ripening at 5° C and at 25° C. In A the data are expressed on the basis of the individual, using the non-after-ripened individual as 100 %. In B these data have been calculated on a cell basis by using the data presented in Figures 4 and 5.

capacity as presented in figure 3 represent changes of a much higher order of magnitude, actually growth visible to the naked eye.

In figure 8 the changes in respiration rate in the various parts of the seed are presented as a function of after-ripening time and temperature. Because of the great difference in absolute amounts, the data are expressed on a percentage basis, using the rates for non-after-ripened seeds as 100 %. For the whole seed, embryonic axis, and individual leaf primordium this basic value is 5.29, 0.246 and 11.1 $\times$ 10^{-3} μl/hr/individual or organ, respectively. Figure 8 A shows that the respiration of whole seeds after-ripened at 5° C increases during after-ripening. However, this increase for whole seeds, a maximum of about 70 %, is small compared to the increase for the embryonic axis and leaf primordia of almost 600 % in 16 weeks. It should be noted that for both the leaf primordia and axis this increase is almost linear with time and does not show a 4 week lag as does the growth capacity and increase in dry weight of the embryonic axis.

In seeds after-ripened at 25° C the respiration rate of whole seeds actually declines to less than half that originally present. The respiration rate of the embryonic axis and leaf primordium does not change significantly with time.

Figure 8 B shows the respiration rate calculated on a cell basis and plotted as a function of after-ripening time and temperature. To do this with whole seeds we have assumed that there are few or no cell divisions in the cotyledons and endosperm, an assumption which is probably valid. Since the axis and leaf primordia are small compared to the rest of the seed, their cell divisions contribute little to the total cell number, and the change per cell on a percentage basis is therefore identical with the value for the whole seed. At 5° C the respiration rate per cell rises for the 1st 8 weeks in both axis and leaf primordium, and thereafter drops in the leaf primordium when most cell divisions occur.

At 25° C after-ripening has little effect on the total oxygen uptake of the axis and leaf primordium cells, with a decrease apparent in the seed as a whole.

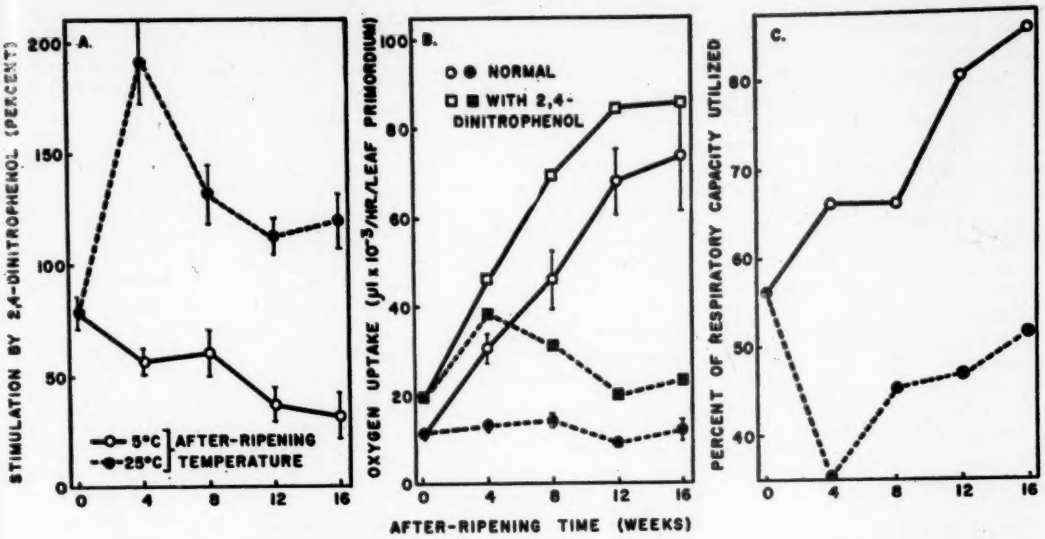
Recognizing the great increase in respiration rate which precedes and accompanies an increase in growth capacity, the question arises as to whether this increase is due to the activation of enzymes previously present, to the synthesis of new enzymes, to the synthesis of other possible rate-limiting factors, or to the transport of such rate-limiting factors from the cotyledons to the growing cells of the embryo. In an attempt to answer these questions, 2,4-dinitrophenol (DNP) was used as an uncoupling agent to obtain an approximate measure of the rate-limiting step in the respiratory chain.

Preliminary experiments with leaf primordia showed that DNP can increase respiration at a concentration of 3 $\times$ 10^{-5} M (fig 7). These experiments also showed that the degree of increase is greater in non-after-ripened than in after-ripened primordia. Therefore, as part of the experiment previously described, DNP at 3 $\times$ 10^{-5} M was added to leaf primordia after the normal respiratory rate had been measured. The results are shown in figure 9. Figure 9 A shows that the amount of increase declines with after-ripening time at 5° C. However, at 25° C during the 1st 4 weeks there is a very sharp increase in DNP action; this is followed by a decline with time. The percent increase of primordia after-ripened at 25° C remained throughout the experiment at least twice as high as that of primordia after-ripened at 5° C.

When the DNP is applied to the leaf primordia, the resulting rate of oxygen uptake might be referred to as "respiratory capacity" and should be a measure of the limiting step in the respiratory chain if substrate and cofactor quantities are not limiting. This respiratory capacity is plotted in comparison with normal respiration rates in figure 9 B. With after-ripening at 5° C both the respiratory rate and capacity rise continuously from the beginning of after-ripening. At 25° C however, the respiratory rate remains approximately constant, but the respiratory capacity increases during the 1st 4 weeks to almost the same level as in material after-ripened at 5° C; thereafter it falls off. Indeed, had a measurement been made at about 3 weeks, it might have shown an identical value at 5° C and at 25° C. Much the same trend is shown in figure 10 with the results calculated on a per cell basis. If we then calculate the actual respiration rate of the leaf primordia as a percentage of their respiratory capacity, it is clear (fig 9 C) that the percent of utilization climbs more or less continuously during after-ripening at 5° C, but at 25° C it declines very sharply during the 1st 4 weeks, thereafter rising somewhat with time but never getting to much over half the degree of utilization shown by the leaf primordia from seeds after-ripening at 5° C. While this experiment was terminated prior to much actual growth, other experiments with more advanced material, such as that shown in figure 7, indicate that this trend for more complete utilization of the respiratory potentialities of the system continues at least into the early stages of active growth.

The fact that the stimulation by DNP declines as the system approaches a growing condition suggests that the rate-limiting step in respiration shifts with the degree of after-ripening and changing growth capacity. This shift seems to be from the phosphorylation system toward a rate-limiting step in the respiratory chain itself, although these data alone might equally indicate a rate limited by respiratory substrate. However, evidence for the respiratory chain as the rate-limiting step is shown in figure 11, where cyanide sensitivity curves for non-after-ripened and after-ripened (15 to 18 weeks at 5° C) leaf primordia are shown. In this case, the after-ripening was carried somewhat closer to actual growth than in the previous case, and the DNP increase was comparable to that shown for after-ripened primordia in figure 7. These data show that after-ripened primordia are more sensitive, both in regard to HCN concentration and total

STIMULATION BY 2,4-DINITROPHENOL (PERCENT)



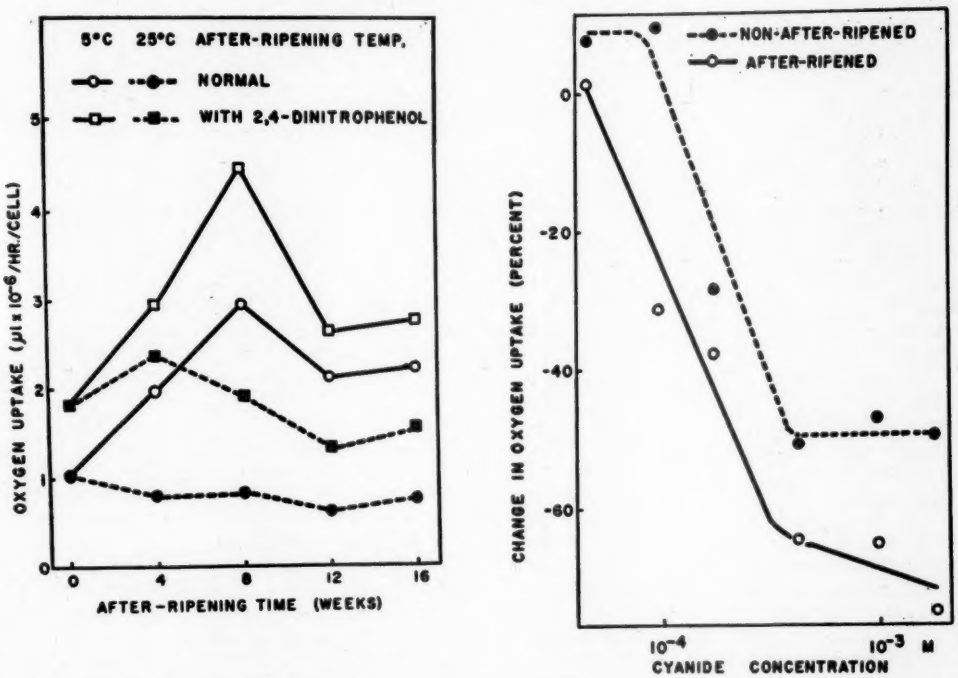


FIG. 9 (*top*). The effect of after-ripening time and temperature on respiration rate and respiratory capacity of embryonic leaf primordia. "Respiratory capacity" is here defined as the maximum respiration rate obtained in the presence of 3×10^{-5} M 2,4-dinitrophenol. The vertical lines represent $\pm$ the standard error of the mean.

FIG. 10 (*lower left*). Changes in respiratory rate and capacity per cell in embryonic leaf primordia as a function of after-ripening time and temperature. These data are calculated from the data presented in figures 5 and 9.

FIG. 11 (*lower right*). The effect of HCN on the oxygen uptake of cherry embryonic leaf primordia from non-after-ripened and from after-ripened seeds.

inhibition, than are non-after-ripened primordia. This is what would be expected if the capacity of the respiratory chain itself became more the limiting factor in respiration.

Discussion

In their recent review article, Toole et al (18) noted that one of the first evidences of the onset of germination is the increase in respiration rate, but that the sequence of enzymatic events has not been established. Thus the data presented in this paper need to be interpreted relative to the normal germination process as well as to the rest period. Indeed, it is possible to consider that the changes which occur slowly during a period of low temperature after-ripening are similar to those which must occur very quickly during the 1st hours of germination in a seed germinating without a rest period.

One of the interesting conclusions from these data is that, for the cherry seed at least, early growth of the embryo is not restricted to cell elongation, but includes cell division. It is particularly interesting to note that in the leaf primordia a significant number of cell divisions occur during the 1st 4 weeks, and occur equally at 5° C and 25° C. At this time the embryo is not capable of any further growth. This might be interpreted to mean that these early cell divisions are independent of the rest period block, possibly a continuation of development which was arrested by seed maturity.

One of the difficulties in interpreting changes relative to the ending of the rest period is the problem of distinguishing cause from result. When the rest period ends and active growth begins, obviously a large number of profound physiological and biochemical changes occur. These are the result of the ending of the rest period. The problem is then to try to set up a timetable of events; those changes preceding the end of the rest period could conceivably be responsible for its ending. For example, the changes in dry weight of the embryonic axis and leaf primordia constitute an interesting problem. Obviously several changes in location and amounts of reserve materials must occur during after-ripening and early growth: 1) Reserves stored in the embryonic axis are utilized in respiration and/or converted to cell wall and protoplasmic materials, and 2) reserves in the cotyledons are used in the cotyledons for respiration and translocated to the growing cells for respiration and synthesis. Since the total dry weight of the leaf primordia is small relative to the axis weight and changes therein, we can, as a first approximation, consider only changes which occur in the embryonic axis. The data in figure 4 C show that the dry weight of the embryonic axis remains constant or drops during the 1st 4 weeks at both 25° C and 5° C, thereafter it remains constant at 25° C but rises steadily at 5° C. Obviously this increase in dry weight at 5° C must indicate translocation of enough materials from the cotyledons to the axis to supply both the requirements for respiration and for synthesis. Apparently much less translocation to the axis occurs at 25° C. Is a block to translocation of nutrients responsible for the rest period? Evidence in the literature (5) suggests that excision of parts of the cotyledons, while making the plants smaller, has no specific effect on breaking the rest period. However, this does not answer the question since it is possible that the stimulus directing translocation arises within the growing cells and that the rest period involves failure of this stimulus. It is equally possible that the failure of the translocation stimulus is just one of many secondary effects of a block elsewhere in metabolism. In other words, we cannot yet distinguish cause from result in this situation.

The most striking changes we have noted during after-ripening have been associated with the respiratory mechanism of the cells. The data show that the respiration rate of both the embryonic axis and the leaf primordia from seeds after-ripened at 5° C rises linearly with time of after-ripening; there is no obvious difference in response between the axis and leaf primordia. It is particularly interesting to note that the respiratory rate increases from the beginning of after-ripening, while the growth capacity rises only after a 4-week lag. Thus the increase in respiration appears to precede the removal of the rest period block. It is particularly interesting to examine the leaf primordia and the changes in respiratory capacity which occur within these 1st critical 4 weeks of after-ripening. At 5° C the respiratory rate and respiratory capacity rise together; this increase is accompanied by division of the equivalent of 51 % of the original cells. At 25° C the cells divide at the same rate, and the respiratory capacity rises almost as rapidly as at 5° C but the respiratory rate fails to increase at all. With further after-ripening the rate and capacity both climb at 5° C but the respiratory capacity declines while the respiratory rate remains constant at 25° C. These changes strongly suggest that the initial effect of after-ripening at 5° C is to increase the efficiency with which the respiratory enzyme system is utilized, hence increasing the supply of energy available for synthesis and growth. In contrast, at 25° C synthesis appears to start normally and then come to a halt for lack of available energy. A somewhat similar situation has been described by Evenari (4) for the lettuce seed.

What does the term "respiratory capacity" mean in the biochemical mechanisms of the cell? Dinitrophenol is usually considered to act by uncoupling mitochondrial oxidations from phosphorylation. If DNP acts to increase respiration, it may therefore be assumed that the rate-limiting step in untreated cells was associated with the supply of phosphate acceptors or their turnover rate. If this is the mechanism by which DNP increases the respiration of cherry embryonic leaf primordia, then the available data strongly suggest that 1 of the changes responsible for an increased respiratory rate in material after-ripened at 5° C is an increased supply of phosphate acceptors or an increased turnover rate. If the former is the

case, then it may be reasoned that the effect of low temperature is to increase the synthesis of phosphate acceptors in the embryonic axis and/or the synthesis and translocation of these acceptors from the cotyledons. As in the case of dry weight changes there is question as to whether this change is cause or result. However, because it occurs earlier than other changes, it is perhaps more likely to be a key step in the breaking of the rest period.

The problem of finding a suitable basis on which to express physiological measurements is a perennial one. In this particular case it is complicated at the outset by the fact that the conversion of storage materials into protoplasmic constituents makes the use of dry weight or total nitrogen somewhat questionable as a basis for expressing data. We have calculated some of the data on a unit cell basis. This is open to question also, 1st because it does not consider the size of the cell and 2nd because it implies an equality among cells which obviously does not exist. The application of this technique to the leaf primordia is probably on a sounder basis than its application to the embryonic axis, since the leaf primordia are more uniformly meristematic and have a much higher proportion of cell divisions. In spite of the difficulties raised, some interesting conclusions result from calculating respiration rate and capacity on a cell basis. For example, assuming that treatment with DNP shifts the rate-limiting step in respiration to the chain of respiratory enzymes, we then have a rough measure of the quantity of enzyme present in the cells. As shown in figure 10, during the 1st 4 weeks after-ripening at 5° C the enzyme must be synthesized (or activated) in a quantity sufficient not only to supply the newly-formed cells but also to increase the quantity within these cells. This increase must continue through the period 4 to 8 weeks when no cell divisions occur. Then from 8 to 12 weeks, when more frequent cell divisions occur, the synthetic rate is not adequate to maintain the rate per cell constant and the respiratory capacity per cell drops. This does not mean that synthesis stops, because the increase in total respiratory capacity per primordium continues to increase at a more or less constant rate; it merely means that cell division and synthesis are not precisely linked.

The changes in respiratory capacity with after-ripening at 25° C are also interesting. Accompanying the cell divisions during the 1st 4 weeks, there would appear to be a considerable amount of synthesis. It is not clear however whether the subsequent decline indicates a destruction of enzyme, its conversion to an inactive form, or a shift to a new rate-limiting step in respiration. It is perhaps significant that the reduction is to, but not below, the original level in non-after-ripened seeds.

In considering the present status of our knowledge of the mechanism of the rest period, it is clear that rest may be considered to involve at least 1 primary block to growth which will have many secondary effects on the blocked cells. Regardless of whether this primary block is in the nature of a chemical inhibitor, a deficiency of some essential hormone or cofactor, or some other type of mechanism, this primary block must operate through the metabolism of the affected cells and, in turn, must be generated and removed by changes in cell metabolism. The problem then is to construct a timetable of metabolic changes and a flow sheet of such compounds as nutrients and hormones sufficiently precise to permit identification of the metabolic block, together with its mode of generation and removal.

Summary

The changes associated with after-ripening in resting cherry seeds have been investigated by utilizing a combination of "conventional" and "ultramicro" analytical methods. With these methods, it has been possible to determine changes in the whole seeds and, more important, in the potentially growing cells of the embryo. In seeds after-ripened at 5° C, the capacity for growth develops linearly with time after a lag of 4 weeks. This capacity for growth is paralleled in the embryonic axis by an increase in dry weight, indicating translocation of material from the cotyledons, but is preceded by cell divisions and a sharp increase in respiration rate. Using 2,4-dinitrophenol to obtain a measure of respiratory capacity, we find that the increase in respiration is paralleled by an increase in respiratory capacity. In contrast, in seeds after-ripened at 25° C growth capacity does not develop and little translocation to the potentially growing cells of the embryo is evident. The respiratory rate remains approximately constant but the respiratory capacity rises, accompanied by a significant number of cell divisions, within the 1st 4 weeks of after-ripening, but thereafter declines to its original level.

We interpret these results as suggesting that one cause of the breaking of the rest period may be the increased availability of energy to the embryo, possibly by an increase in the supply of phosphate acceptors. Whether this is the primary reaction responsible for breaking the rest period or just one of many secondary reactions which follow activation of the cells is not clear.

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FORMATION OF β-PHENYLGLUCOSIDE IN PLANT LEAVES[1, 2]

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The formation of phenol sulfate or phenylglucuronide and subsequent excretion of these products has been observed on feeding phenol to animals (1). This paper reports the formation of β-phenylglucoside on feeding traces of phenol to plant leaves. Recently similar results have been reported on the formation of other phenolic glucosides (3).

MATERIALS AND METHODS

Uniformly labeled glucose-C^{14} or sedoheptulose-C^{14} solutions with specific activity of 3.7 μc/mg of carbon were obtained by biosynthesis and isolated by paper chromatography (5). The solution of sedoheptulose-C^{14} contained 1.4 mg/ml and that of glucose-C^{14}, 0.7 mg/ml. Synthetic glucose 1-C^{14} and 6-C^{14} were obtained from the National Bureau of Standards, and solutions of these sugars were prepared by dissolving 5 mg of the sugar in 5 ml of water.

Synthetic phenylglucoside was obtained by the method described by Helferich and Schmitz-Hillebrecht (2). The position of phenylglucoside on the chromatograms was determined by placing the chromatogram over a calcium tungstate screen and irradiating it with ultraviolet light (2537 Å maximum). The phenylglucoside appeared as a blue-violet spot on the chromatogram and as a dark shadow on the screen.

Barley or wheat leaves were fed the solutions of labeled sugar by placing the leaf base in a tube with 100 μl of the sugar solution with or without phenol. After the leaf had taken up this solution, a continual supply of water was provided. At the end of the experiment, the leaves were ground in liquid nitrogen, and the powder was taken up in water and immediately heated in a boiling water bath. After separation of the supernatant liquid from the solid debris, the extract was concentrated, and its components separated by the chromatographic procedures previously used (4). The C^{14}-labeled compound suspected of being phenylglucoside was separated by these procedures and located on the chromatograms by radioautographic techniques. The area was cut out and eluted with distilled water. The eluate was then again chromatographed in an ethyl alcohol : 15 % acetic acid (8 : 1 by volume) solvent system in order to separate any contaminating phenol. The C^{14}-labeled compound (phenylglucoside) was then eluted from the chromatograms, and its ab-

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[2] A part of this paper has been taken from the dissertation submitted by C. W. Nystrom to the University of Oklahoma in partial fulfillment of the requirements for the Ph. D. degree in chemistry, 1956.

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sorption spectrum was determined in the ultraviolet region before and after hydrolysis with 2.0 *N* HCl. The effect of emulsin on the eluate was determined by adding a small amount of emulsin and chromatographing the mixture after allowing it to stand for 15 minutes.

Results

When synthetic glucose 1-C^{14} or 6-C^{14} was fed to plant leaves, only traces of C^{14} activity could be detected on the chromatograms in the area occupied by synthetic phenylglucoside (R_f 0.80 in phenol : water and 0.65 in butanol : propionic acid : water solvent systems). When 60 μg of phenol was added to 100 μl of these glucose-C^{14} solutions before feeding to the plant leaves, 3 to 7 % of the total radioactivity fed to the plant was converted into a C^{14}-labeled compound that occupied the same position as phenylglucoside on the chromatograms. Further purification of the C^{14}-labeled compound, formed when phenol was added to the sugar solution, was achieved by eluting the compound and rechromatographing. Radioactivity of the labeled compound co-chromatographed with synthetic phenylglucoside in all solvent systems used. An absorption spectrum of eluates from chromatograms containing the labeled compound was identical to that of synthetic phenylglucoside. After hydrolysis with 2 *N* HCl, the absorption spectrum was identical to that of hydrolyzed phenylglucoside. The only C^{14}-labeled product present after hydrolysis was shown by co-chromatography with authentic glucose to be glucose-C^{14}. The compound was also hydrolyzed by emulsin, and the only C^{14}-labeled product found was glucose-C^{14}. Since emulsin is specific for β-glycosidic bonds, the labeled compound is a β-phenylglucoside.

If solutions of either biosynthetic sedoheptulose-C^{14} or glucose-C^{14} were extracted with ether before they were fed to the plant, no phenylglucoside was formed. Addition of 60 μg of phenol to the ether-extracted sugar solutions before feeding the labeled sugars to the plants resulted in formation of substantial amounts of the C^{14}-labeled phenylglucoside. Three hundred micrograms of phenol completely inhibited metabolism of the sugars by the plant leaves, and hence no C^{14}-labeled phenylglucoside was formed. C^{14}-labeled phenylglucoside was formed when the plant leaf was fed either a solution of biosynthetic glucose-C^{14} or sedoheptulose-C^{14} just as the sugar solution had been isolated by elution of the C^{14}-labeled sugar from chromatograms with water. The labeled phenylglucoside formed with biosynthetic sedoheptulose-C^{14} solution was found to be identical to an unidentified C^{14}-containing compound (R_f 0.80 in phenol : water and 0.65 in butanol : propionic acid : water) previously reported in studies on metabolism of sedoheptulose-C^{14} in plant leaves (4).

Discussion

When sedoheptulose-C^{14} or glucose-C^{14} was fed to leaves with small amounts of phenol, β-phenylglucoside was formed, but there was no evidence for the formation of a phenylheptuloside. Apparently, the sedoheptulose-C^{14} was first converted to glucose-C^{14}, which was then utilized in the formation of the phenylglucoside-C^{14}. This is in agreement with our present knowledge that no glycosides of heptulose have been observed in natural products. A compound previously reported as unidentified in studies on sedoheptulose metabolism (4) was found to be β-phenylglucoside and apparently resulted from action of plant enzyme systems on traces of phenol in the biosynthetic sugar-C^{14} solutions prepared by chromatographic techniques. It was shown (4) that the unidentified compound now known to be β-phenylglucoside was formed in etiolated as well as green plant leaves with or without light. These results show that the plant leaves have enzyme systems capable of catalyzing the formation of β-phenylglucoside from phenol and glucose under a variety of environmental and physiological conditions.

As a note of caution, the experimental results illustrate the potential danger of contamination with phenol of solutions of biosynthetic C^{14} compounds that are isolated by paper chromatographic techniques in which a phenol solvent system is used. If phenol vapors are present in the room, chromatograms will become contaminated even while drying. Traces of phenol on the chromatogram will not disappear on long standing and are sufficient to cause biological reactions, such as glycoside formation, or even in some cases wilting of leaves. Exhaustive ether extraction of acidified eluate from chromatograms will remove the phenol. Passage of the eluate through Dowex-1 or charcoal not only removes any contaminating phenol but also removes traces of yellow impurities found in the concentrated eluate.

Summary

Leaves form β-phenylglucoside-C^{14} under a variety of environmental and physiological conditions when fed phenol and glucose-C^{14} or phenol and sedoheptulose-C^{14}. No glycoside of sedoheptulose was detected. Sufficient amounts of phenol may be present in solutions of biosynthetic sugar-C^{14} isolated by paper chromatography involving phenol solvent systems to result in the production of substantial amounts of β-phenylglucoside-C^{14} when the sugars are fed to the plant.

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OXIDATION OF INDOLEACETIC ACID BY QUACKGRASS RHIZOMES[1,2]

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The enzymatic oxidation of 3-indoleacetic acid (IAA) which was first described in detail by Tang and Bonner (16, 17), has been observed in several types of plants, e.g., fungi (13), ferns (14), monocotyledonous plants (20), and dicotyledonous plants (16, 17, 19). Although differences have been observed in regard to the enzyme's requirement for a phenolic cofactor, and in the pH optimum, the requirement for manganese seems to be general.

Interest in the IAA oxidase of quackgrass rhizomes arose from the finding that the rhizomes showed a seasonal variation in bud activity (5). The hypothesis that the bud activity of quackgrass rhizomes may reflect the concentration of IAA in the rhizomes, and that this concentration may be controlled by IAA oxidase was considered. Others have presented evidence both for (4) and against (2) the participation of IAA oxidase in the control of IAA concentration under physiological conditions. In the course of the present study, some characteristics of the IAA oxidase of quackgrass rhizomes have been compared with the IAA oxidase from other sources. The relationship between total nitrogen of the rhizome tissue, IAA oxidase activity, and the protein content of the enzyme extracts is discussed.

MATERIALS AND METHODS

Rhizomes were obtained from an undisturbed, heavily infested quackgrass sod at weekly intervals during the growing season beginning March 27, 1957. The rhizomes were cut into 20 mm sections, each having 1 bud. These sections were quick frozen in liquid air and lyophilized. The dried sections were ground to 60 mesh size with a Wiley mill, and the resulting powder was stored in a desiccator over phosphorus pentoxide at −15° C until needed.

The enzyme preparations for oxidizing IAA were obtained from lyophilized quackgrass rhizome material by extracting 2 g of the stored powder with 20 ml of distilled water at 5° C for 24 hours. The mixture was centrifuged at 9000 × G for 30 minutes and the supernatant decanted. The residue then was extracted with 10 ml of water for 12 hours; the mixture was centrifuged and the supernatant decanted. The residue finally was washed with 3 ml of water. The combined extracts were dialyzed either against running tap water at 10° C for 40 hours and then distilled water at 5° C for 10 hours, or against several changes of distilled water at 5° C for 48 hours. The dialyzed extracts were made up to a standard volume of 40 ml.

Enzyme preparations were obtained from fresh quackgrass rhizomes by grinding the rhizomes with ice in a Nixtamal mill. The milled material was pressed through cheesecloth, and the collected liquid was centrifuged at 3000 × G for 30 minutes. Solid ammonium sulfate was added to the decanted supernatant to make the solution saturated. The precipitate obtained was redissolved in distilled water, and solid ammonium sulfate was added to this solution to make it 80 % saturated. The precipitate obtained was dissolved in distilled water and dialyzed for 24 hours against running tap water at 10° C and finally against distilled water at 5° C. This preparation was centrifuged, and the clear supernatant was used as the enzyme solution.

Peroxidase activity was measured by following the formation of purpurogallin colorimetrically at 430 mμ with a Bausch and Lomb Spectronic 20 colorimeter. The reaction mixture, in a 100 × 12 mm test tube, consisted of 0.5 ml of 0.1 M phosphate buffer at pH 6.0, 0.5 ml 0.01 M pyrogallol, 0.1 ml of 0.15 % H_2O_2, 0.5 ml of enzyme solution, and water to make a total volume of 3.0 ml. The reaction was started by the addition of the enzyme. Readings were taken at 10 second intervals and the initial rate calculated from the readings of the 1st minute.

The oxidation of manganese was measured by following the formation of manganipyrophosphate spectrophotometrically. Manganipyrophosphate has an absorption peak at 258 mμ (7). Measurements were made in a 1 cm cuvette with a Beckman DU spectrophotometer at 258 mμ. The reaction mixture consisted of 0.5 ml of 0.1 M pyrophosphate at pH 7.0, 0.5 ml of 0.01 M $MnCl_2$, 0.5 ml of 0.001 M resorcinol, 0.1 ml of 0.15 % H_2O_2, 1.0 ml of enzyme solution, and water to make a total volume of 3.5 ml. The reaction was started by addition of the enzyme. Readings were taken at 30 second intervals, and the initial rate was calculated from the readings of the 1st 2 minutes.

The IAA oxidase activity was determined by measuring oxygen uptake at 30° C in a Warburg respirometer (18). The volume of the reaction mixture was 3.0 ml; 0.15 ml of 20 % KOH and a filter paper wick were present in the center well. The reaction was started by the addition of either the enzyme or IAA from the side arm.

The protein content of the enzyme solutions was determined by the method of Lowry et al (8). Reducing sugars were determined by the method of Park and Johnson (10). Total nitrogen content of the powdered, lyophilized rhizome samples was determined by the Kjeldahl procedure. Horseradish peroxidase (HRP) was obtained from Nutritional Biochemicals Corporation. HRP solutions were made by dissolving 10 mg of the powder in 100 ml distilled water.

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[2] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

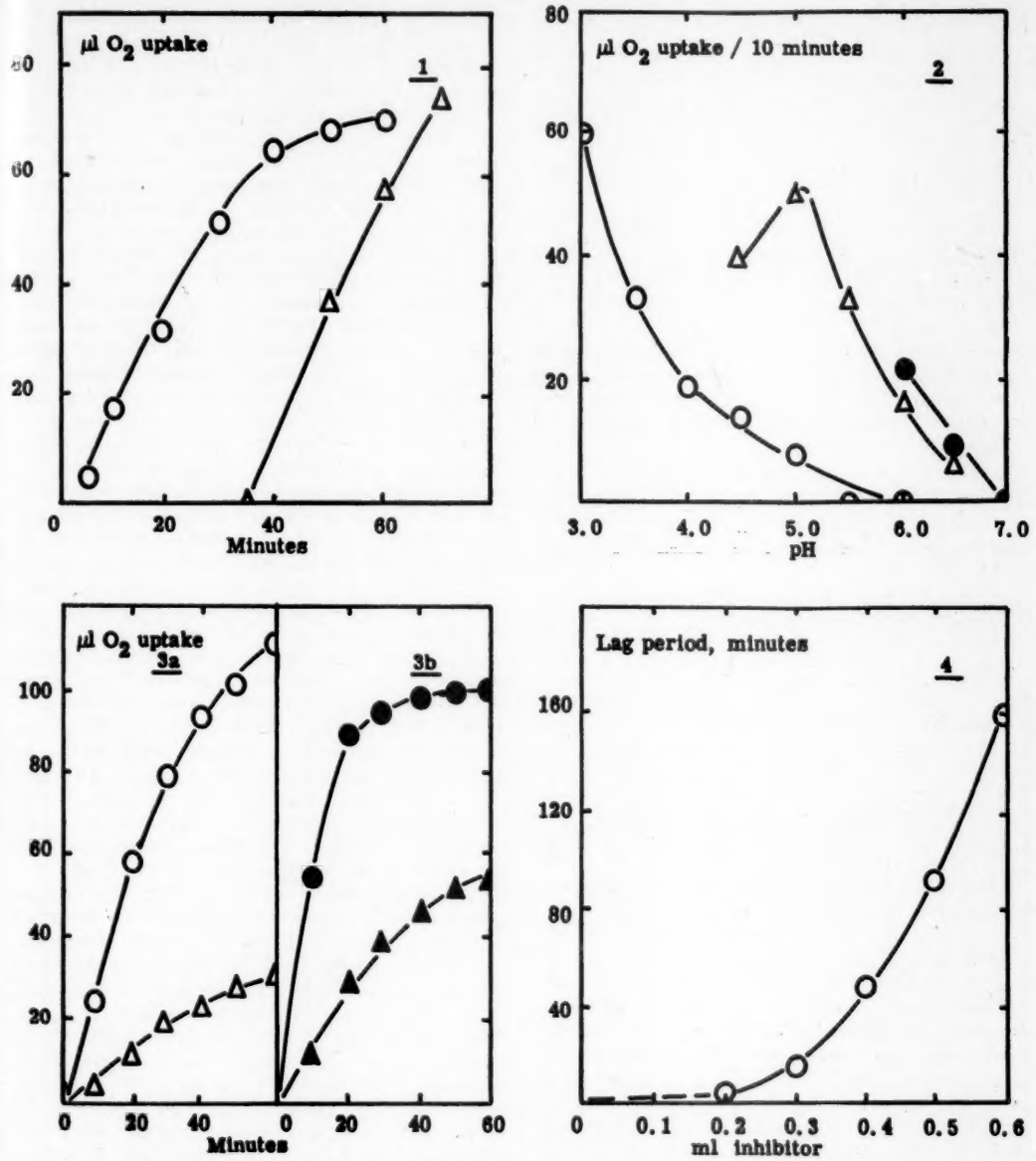

FIG. 1. Effect of dialysis on an IAA oxidizing preparation from quackgrass rhizomes. The reaction mixture contained 0.5 ml 0.1 M phosphate buffer, pH 6.0; 0.3 ml 0.01 M $MnCl_2$; 0.3 ml 0.01 M resorcinol; 1.0 ml 0.01 M IAA; 0.5 ml enzyme solution. △, before dialysis; ○, after dialysis.

FIG. 2. Effect of buffer and pH on IAA oxidation by quackgrass rhizome preparations. The reaction mixture contained 0.5 ml 0.1 M buffer; 0.3 ml 0.01 M $MnCl_2$; 0.2 ml 0.01 M resorcinol; 0.5 ml 0.01 M IAA; 1.0 ml enzyme solution. (The pH range tested with phosphate buffers was limited, because they buffer poorly in the region directly below pH 6.) ○, citrate; △, succinate; ●, phosphate.

FIG. 3. Effect of cofactors on the oxidation of IAA by quackgrass rhizome preparations. The complete reaction mixture for 3 a was the same as in figure 2; phosphate buffer pH 6.0. In 3 b resorcinol was replaced by an equal amount of 2,4-dichlorophenol. △, enzyme + IAA + resorcinol; ○, enzyme + resorcinol + IAA + Mn^{2+}; ▲, enzyme + 2,4-dichlorophenol + IAA; ●, enzyme + 2,4-dichlorophenol + Mn^{2+} + IAA. Oxygen uptake in the absence of phenolic cofactor was negligible.

FIG. 4. Inhibition of the IAA oxidizing activity of HRP by the naturally occurring inhibitor from quackgrass rhizomes. The reaction mixture contained 0.5 ml 0.1 M phosphate buffer, pH 6.0; 0.3 ml 0.01 M $MnCl_2$; 0.2 ml 0.01 M resorcinol; 0.5 ml 0.01 M IAA; 0.2 ml HRP solution; rhizome extract.

Results and Discussion

The presence of an IAA oxidizing enzyme in quackgrass rhizomes was demonstrated readily. Extracts from powdered, lyophilized quackgrass rhizomes were found to contain a dialyzable inhibitor(s) of the enzyme, as shown in figure 1. Subsequently all preparations were dialyzed for at least 48 hours before assay.

The efficiency of the extraction procedure for the lyophilized rhizome powders was tested. Two grams of the powder was extracted for 12 hours with 20 ml distilled water. The supernatant was decanted after centrifuging at 9000 × G for 30 minutes. Five more similar extractions were made with 10 ml portions of water. The 6 samples were dialyzed for 48 hours before assay. The results (table I) show that all of the enzymatic activity was obtained in 3 extractions.

Table I

Enzyme Extraction from Powdered, Lyophilized Quackgrass Rhizomes

Extract	Protein, μg/ml	Mn oxidase, Peroxidase, ΔO.D./min.	ΔO.D./10 sec	IAA oxidase, μL O_2/10 min
1	375	0.060	0.040	7.0
2	220	0.025	0.015	2.5
3	121	0.007	0.007	0
4	56	0	0	0
5	47	0	0	0
6	36	0	0	0

The reaction mixture for the oxidation of IAA contained 1.0 ml of enzyme extract; 0.5 ml 0.1 M phosphate buffer pH 6.0; 0.3 ml 0.01 M $MnCl_2$; 0.2 ml 0.01 M resorcinol; 0.5 ml 0.01 M IAA.

Other assays and analyses were made as described in Materials and Methods.

The effect of pH on the oxidation of IAA by the enzyme preparation from fresh quackgrass rhizomes was tested with 3 buffers. The results with orthophosphate and succinate buffers agreed in the pH range where both were tested. A pH optimum of about 5 is indicated with succinate. With citrate buffer, the apparent pH optimum is much lower (fig 2). Attention has been drawn to the chelation of manganic ions by citrate (6); a pH optimum for IAA oxidase determined with citrate buffer is invalidated by chelation. Appreciable activity in citrate buffer should be observed only at low pH where the undissociated acid and the monoanion, which do not chelate, are the predominant forms. The acid pH optima reported for some IAA oxidase preparations may be attributed to the use of citrate buffer (12). Chelating effects of buffers used may explain some of the differences in pH optima found for IAA oxidase preparations from different sources.

The cofactor requirements of the IAA oxidizing enzyme of quackgrass rhizomes appear to be the same as those for the enzymes found in wheat leaves (9 and in lupine hypocotyls (15). The effect of cofactor on a preparation from fresh rhizomes is shown in figure 3. The cofactors had the same relative effec on enzymes from powdered, lyophilized rhizomes.

The naturally occurring inhibitor(s), which is hea stable, causes a lag period in oxidation of IAA. A crude inhibitor preparation was obtained by boilin 2 g of the powder with 20 ml of distilled water. Th supernatant was decanted and the extraction repeate 3 times with 10 ml portions of hot water. The inhibitor content of the combined extracts was assayed by measuring its effect on the oxidation of IAA by a HRP preparation. Figure 4 shows that the lag period induced increases disproportionately (approximately exponentially) as the concentration of inhibitor is increased. The lag period may be interpreted as arising from the inactivation of an essential intermediate by the inhibitor. This could result in the concomitant inactivation of the inhibitor. After the concentration of the inhibitor has been reduced to an innocuous level, the reaction should be free to proceed. A lag period in the oxidation of IAA has been observed with the naturally occurring inhibitor from wheat leaves and with catechol (9). A lag period is also found with agents which chelate manganese, such as citrate and pyrophosphate.

In March 1958, after a minimum of 7 months storage of the powdered, lyophilized quackgrass rhizomes, it was found that even prolonged dialysis of their extracts did not remove all of the naturally occurring inhibitor of IAA oxidase. However, no inhibition of peroxidase and manganese oxidase activities was observed. Ray (11) has discussed the role of manganese in the enzymic oxidation of IAA. According to one theory (9), the oxidation depends on the formation of manganic ions. If this is the case, the inhibition by the naturally occurring inhibitor must take place in the reaction sequence after the formation of manganic ions. The inhibition of the endogenous IAA oxidase from several dialyzed sample extracts is shown in figure 5. Extracts from samples harvested later in the growing season showed longer lag periods even though the powders had been stored for a shorter length of time when the determination was made. The same samples caused a lag period in IAA oxidation as shown in figure 6. After the lag period was overcome, the sum of the activities of the endogenous enzyme and the HRP gave greater rates of oxygen uptake than that observed with HRP alone.

After prolonged dialysis, the extract might be expected to contain polysaccharide in addition to protein. The principal polysaccharide of quackgrass rhizomes has been shown to be a complex fructosan (1), which has been called triticin. Triticin was obtained from extracts showing a non-dialyzable inhibitor by a modification of the method of Arni and Percival (1). One hundred ml of the extract was treated with basic lead acetate and the precipitate removed by filtration. Hydrogen sulfide was bubbled through the filtrate to precipitate the lead, and then air was bubbled through

to remove excess hydrogen sulfide. The lead sulfide was removed by filtration, and the filtrate was concentrated to 10 to 15 ml at 40° C and a pressure of 15 mm Hg. The triticin was precipitated by pouring this solution into 300 ml ethanol, and was recovered by filtration. The triticin was re-precipitated 3 times from water with ethanol. The product was washed with ether and dried in a vacuum desiccator. As previously described (1), the triticin was hygroscopic, and in solution it showed no reducing power. A 2 % solution gave a specific rotation of −42°. After hydrolysis in 0.1 *N* HCl in a 55° C water bath for 30 minutes, the specific rotation was −72°. This value was not changed on further heating.

The purified triticin did not inhibit the oxidation of IAA by HRP, indicating that the polysaccharide itself is not the non-dialyzable inhibitor. Glucose and fructose were also tested and did not inhibit the enzymic oxidation.

It is probable that the inhibitory substances are bound to high molecular weight compounds and so are non-dialyzable. Perhaps fresher material contains an enzyme, lost on aging, which can release the inhibitory substances, and in extracts from aged material, devoid of the enzyme, the inhibitor is not released in a dialyzable form.

A cold water extract of powdered, lyophilized quackgrass rhizomes was hydrolyzed with 0.1 *N* HCl and extracted with several portions of ether. The ether was removed by evaporation and the residue dissolved in water. The ether soluble and water soluble fractions were compared with the unfractionated hydrolyzed sample. Most of the inhibitor remained in the aqueous phase. A similar fractionation was performed with an inhibitor preparation obtained by extraction with hot water, and a similar distribution of the inhibitor was observed. This behavior is comparable to that observed with the naturally occurring inhibitor of wheat leaf IAA oxidase (9).

The protein content of the IAA oxidase preparations paralleled the total nitrogen content of powdered, lyophilized rhizome tissue, indicating that changes in IAA oxidase were merely part of a general change in nitrogenous constituents. Further determinations showed that though the total IAA oxidase activity of the rhizomes changed with the season, the specific activity per unit of nitrogen remained virtually constant during these seasonal changes. The total IAA oxidase activity was directly related to the rates of oxidation measured, since a standard volume of extract was obtained from a standard weight of powder. The reduction in IAA oxidase activity as the season progressed was simultaneous with the increase in inhibitor. If these 2 factors are effective in vivo there will be less IAA oxidation as the season progresses.

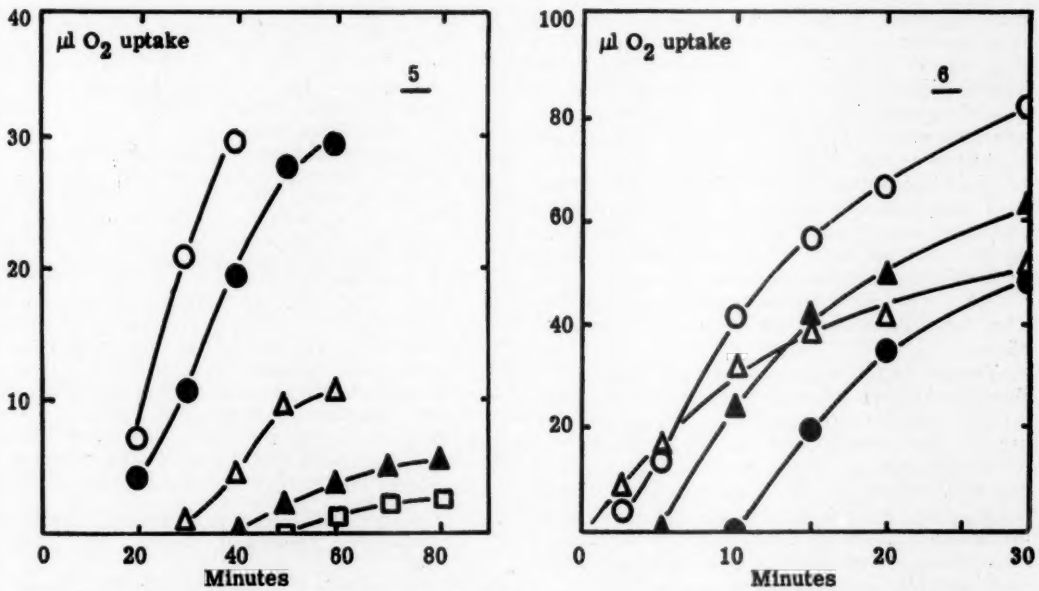

FIG. 5. Inhibition of endogenous IAA oxidizing activity of extracts from lyophilized and powdered rhizome samples by a non-dialyzable inhibitor. Sampling dates: ○, 4–3; ●, 4–24; △, 5–22; ▲, 6–5; □, 8–7. Reaction mixtures as in table I.

FIG. 6. Inhibitory effect on IAA oxidizing activity of HRP of extracts from powdered, lyophilized rhizomes showing non-dialyzable inhibition. △, HRP alone; ○, HRP + extract from sample taken 4-3; ▲, HRP + extract from sample taken 5–22; ●, HRP + extract from sample taken 8–7.

It is interesting that the changes in IAA oxidase, manganese oxidase, and peroxidase correspond. This is a further indication that IAA oxidase activity is a facet of peroxidase activity, as suggested by Galston et al (3) and later by Stutz, (15), and depends on the peroxidatic oxidation of manganese (table II).

TABLE II

ENZYMIC ACTIVITIES OF EXTRACTS FROM POWDERED, LYOPHILIZED QUACKGRASS RHIZOMES

SAMPLING DATE	% TOTAL N IN DRIED SAMPLE	PROTEIN μG/ML	Mn OXIDASE ΔO.D./MIN	PEROXIDASE ΔO.D./10 SEC	IAA OXIDASE μL. O_2/10 MIN	IAA OXIDASE QO_2 (N)
4–3	0.68	465	0.060	0.025	14.0	1,130
4–24	0.58	310	0.040	0.020	10.0	1.210
5–22	0.24	245	0.030	0.010	7.5	1,100
6–5	0.20	205	0 023	0.010	6.0	1,100
8–7	0.37	290	0.028	0.010	7.5	970

SUMMARY

Quackgrass rhizomes contain an IAA oxidase whose characteristics and cofactor requirements, for manganese and a phenolic compound, are similar to those reported for the enzyme from other plant sources. The rhizomes contain an inhibitor(s) which causes a lag period in the oxidation of IAA. The length of the inhibition period increases disproportionately (approximately exponentially) as the concentration of the inhibitor is increased. It was possible to remove the inhibitor from extracts of fresher samples of lyophilized rhizomes by dialysis; extracts from aged samples retained the inhibitor even after prolonged dialysis. The inhibition does not appear to be associated with the complex polysaccharide present in the rhizomes or its monosaccharide components. The inhibitor remained in the aqueous phase when solutions of the inhibitor were extracted with ether.

The total IAA oxidase activity of the quackgrass rhizomes showed a seasonal variation, but the specific activity remained virtually constant during the same period. The data indicate that variations in IAA oxidase activity are closely associated with general changes in the nitrogenous constituents of the rhizome tissue. The decrease in IAA oxidase activity and increase in inhibitor take place at the same time. If these effects are operative in vivo, there will be less IAA oxidation as the season progresses.

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MALONATE AS A PARTICIPANT IN ORGANIC ACID METABOLISM IN BUSH BEAN LEAVES[1]

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Interest in malonic acid has been largely directed towards its role as a competitive inhibitor of succinic dehydrogenase. Very little attention has been directed toward the occurrence of malonic acid in plants or toward the metabolism of malonic acid per se.

Bentley (1) recently showed malonate to be present in some 18 species of legumes, one of which had concentrations as high as 2 mg/g dry weight. Malonic acid has also been reported in species from families other than the legume family (1, 13, 20).

That malonic acid could be metabolized was established over 30 years ago when it was shown that malonate could serve as the sole carbon source for certain bacteria (3). More recent work established that malonate was readily metabolized by enzyme preparations from fungi (5, 6, 7, 10, 17, 18, 19), mammals (8, 11, 12), and peanut leaves (4).

Recently Rhoads (16) and the present authors (21) reported that malonate was one of the major organic acids in bush bean leaves, often reaching levels as high as 10 mg/g dry weight.

Incubation of malonate with a particulate preparation from bush bean leaf tissue suggested that malonate was metabolized. This paper describes experiments undertaken to study the metabolism of malonate in bush bean leaves, the cofactors required, and the pathway involved.

MATERIALS AND METHODS

For each respiration experiment to be discussed, a particulate preparation was isolated from approximately 60 g fresh weight bush bean leaf tissue (*Phaseolus vulgaris*, var. Stringless Pod). All enzyme preparation procedures were conducted at 0° C. The leaf tissue was ground by mortar and pestle with 0.25 M sucrose in 0.1 M potassium phosphate buffer, pH 7.0. The homogenate, after filtering through cheesecloth, was spun at 1000 × G and the supernatant then centrifuged at 30,000 × G for 10 minutes. The resulting precipitate was washed 3 times and the final precipitate recovered in 10 ml of the above buffer. One ml of this preparation was added to each Warburg vessel, thus providing each vessel with the particulate fraction from approximately 6 g fresh weight leaf tissue. Respiration studies were conducted in an air phase at 30° C and the data expressed as μl O_2 consumed or CO_2 evolved per hour per flask. Carbon dioxide data were obtained by the double flask method. Quantities of cofactors employed in the respiration studies are listed in table I. The top 3 cofactors, CoA, ATP, GSH, and a metal have been reported necessary in the metabolism of malonate (4, 6, 11, 12, 17, 18, 19). To obtain an active Krebs cycle system DPN, TPN, lipoic acid, TPP, cytochrome C, and OAA were also included.

TABLE I

QUANTITIES OF COFACTORS EMPLOYED IN METAPOLISM STUDIES

COFACTOR*	μM
CoA	1.0
ATP	4.9
GSH	5.4
$MnSO_4 \cdot H_2O$	7.0
$MgSO_4 \cdot 7\ H_2O$	16.0
DPN	0.5
TPN	0.05
Lipoic acid	0.1
TPP	6.0
OAA	2.0
Cytochrome C	0.02

* Abbreviations: CoA = coenzyme A; ATP = adenosine triphosphate; GSH = reduced glutathione; DPN = diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide; TPP = thiamine pyrophosphate; OAA = oxalacetic acid.

Quantitative organic acid separation was accomplished by silica gel chromatography using the method of Lees and Kuyper (9), and by ion exchange chromatography using the methods of Palmer (14) as modified by Rhoads (15). One dimension paper chromatography, ether : HAc : H_2O solvent (5 : 2 : 1), was used to separate organic acids following incubation with labeled malonate. Whatman no. 1 paper was employed and the spots were identified by comparison to authentic standards and published R_f values. The resulting spots were counted on a gas flow counter. The specific activity of the 2-C^{14} malonate employed was approximately 1000 cpm/μM (assayed as malonic acid).

RESULTS AND DISCUSSION

Table II lists the quantities of organic acids separated from bush bean leaves. It may be noted that

TABLE II

QUANTITIES OF ORGANIC ACIDS ISOLATED FROM BUSH BEAN LEAVES*

ORGANIC ACID	μEG/G DRY WEIGHT
Malate	448
Citrate + isocitrate	229
Malonate	128
Fumarate	48
Succinate	45

* Organic acids isolated by ion exchange chromatography (14, 15).

[1] Received October 3, 1958.

malonate was present in very large amounts and was 1 of the 3 major organic acids. Other acids isolated were fumarate, succinate, malate, and citrate. These data indicated the presence of 4 of the Krebs cycle acids and suggested the existence of the Krebs cycle oxidation scheme in bush bean leaves.

To determine if the Krebs cycle enzymes were operative in the particulate preparation, 40 μM of the various Krebs cycle acids were incubated individually with the enzyme preparation (table III). The oxidation rates of the various substances tested suggested the presence of the enzymes necessary for the operation of the Krebs cyle. That the entire Krebs cycle was operative was indicated by a substantial rate of pyruvate oxidation.

TABLE III

OXIDATION RATES OF KREBS CYCLE ACIDS BY LEAF PARTICULATE FRACTION

SUBSTRATE*	μl O_2/HR/VESSEL
Citrate	498
Isocitrate	462
α-Ketoglutarate	560
Succinate	532
Fumarate	732
Malate	535
Pyruvate	530

* Substrate concentration = 40 μM.
Endogenous rate = O.

Nakada (11, 12), working with rat tissues, and Giovanelli and Stumpf (4), with peanut leaves, showed that the enzymes involved in malonate metabolism in those tissues were located in the mitochondria. To determine if the particulate fraction of bush bean leaves was capable of metabolizing malonate, 60 μM of 2-C^{14} labeled malonate were incubated with the enzyme preparation for 90 minutes. The incubation mixture was inactivated with 6 N H_2SO_4 and the organic acids separated by paper chromatography (table IV). Substantial amounts of radioactivity were detected in the citrate-isocitrate spot and in the malate spot. Trace amounts were also present in succinate and fumarate. These data indicated that the particulate preparation was capable of metabolizing malonate, and that the number 2 carbon of malonate was incorporated into the Krebs cycle acids.

TABLE IV

DISTRIBUTION OF C^{14} IN KREBS CYCLE ACIDS AFTER 90-MINUTE INCUBATION WITH 2-C^{14} MALONATE

KREBS CYCLE ACID	CPM *
Citrate + isocitrate	543
Malate	1030
Succinate	Trace
Fumarate	Trace

* Counts per minute determined by counting acid spot on chromatography paper in gas flow counter.

Recent work by Hayaishi (6), Wolfe et al (18), and Gray (5), all working with *Pseudomonas fluorescens*, and Nakada et al (12) with rat tissue, have demonstrated that malonate was decarboxylated to acetate and CO_2 as follows:

$$\text{Malonate} \xrightarrow{\text{ATP,CoA}} \text{Malonyl CoA} \xrightarrow{Mg++} \text{Acetyl CoA} + CO_2$$

The cofactors required for this decarboxylation were shown to be CoA, ATP, GSH, and Mg. Malonate was first converted to malonyl CoA, the reaction requiring ATP. Decarboxylation of malonyl CoA to acetyl CoA was shown to require Mg. Acetyl CoA was then available to participate in fat metabolism or condense with OAA and enter the Krebs cycle oxidation scheme.

In an effort to establish that acetate was a participant in malonate metabolism in bush bean leaves, 60 μM of 2-C^{14} labeled malonate were incubated for 3 hours with a particulate preparation. The incubation was conducted in a nitrogen atmosphere and the cofactors added were CoA, ATP, GSH, and Mn. The Krebs cycle cofactors and OAA were omitted to facilitate the isolation of radioactive acetate. Following the 3-hour incubation, the particulate fraction in the mixture was centrifuged at 30,000 × G and the supernatant lyophilized and chromatographed on a

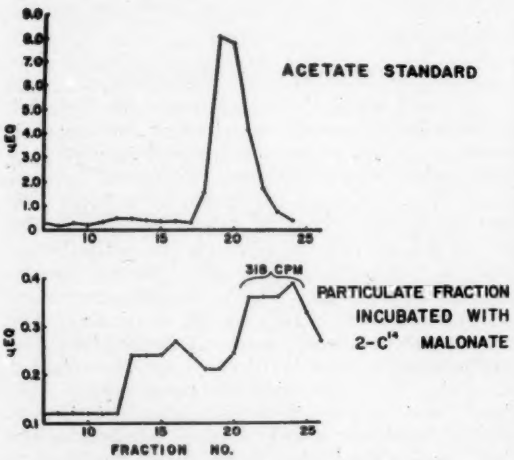

FIG. 1 (*upper graph*). Isolation of authentic acetate by silica gel chromatography.
(*lower graph*). Isolation of labeled acetate from particulate preparation incubated with 2-C^{14} malonate.

silica gel column (fig 1). The lower graph presents titration values of the effluent fractions from the reaction mixture, while the upper graph presents titration values of the effluent fractions from authentic acetate. The peak volume of the acid from the reaction mix-

ture, while shifted slightly to the right, corresponded quite closely with the peak volume of authentic acetate. Effluent fraction numbers 19 to 26 of the reaction mixture were concentrated and the radioactivity determined. The combined fractions were shown to have 318 cpm. All preceding fractions, including the small peak between fractions 12 and 18, were found to be void of radioactivity. The radioactive concentrate from fractions 19 to 26 was then rechromatographed on paper against authentic acetate, using NH_4 : isopropanol solvent (60 : 40). Identical R_f values were obtained and radioactivity was detected in the spot from the incubated material. These data indicated that the labeled acetate identified in the reaction mixture resulted from the anaerobic metabolism of 2-C^{14} malonate.

To establish the cofactor requirements for malonate metabolism in bush bean leaves, 60 μM of malonate were incubated with a particulate preparation. The incubation was conducted in an air phase and the cofactors added were those listed in table I. Table V

TABLE V

EFFECT OF COFACTOR OMISSION ON MALONATE DECARBOXYLATION RATE

TREATMENT	μl CO_2/HR/VESSEL
Complete	92
−CoA	19
−ATP	56
−GSH	44
−metal	20

Substrate = 60 μM malonate.
Endogenous rate = O.

presents the decarboxylation rates in the complete system and in the absence of individual cofactors. In the complete system, the rate of CO_2 evolution was 92 μl/hr/vessel. In the absence of CoA, ATP, GSH, or metals the rate was substantially reduced, thus indicating the necessity of these cofactors. Furthermore, when OAA was omitted from the oxidation reaction, a 50 % reduction of oxygen consumption was obtained. This reduction suggested the necessity of OAA for condensation with the acetate produced from the malonate substrate.

To study the metal requirement in malonate decarboxylation, 60 μM malonate were incubated in the presence of 4 different metals and the decarboxylation rates determined (table VI). It may be noted that the addition of Mn^{++} to the reaction mixture resulted in the highest rate of decarboxylation. The addition of Mg^{++}, Fe^{++}, or Zn^{++} failed to effect a significant decarboxylation of malonate. Using a particulate preparation from peanut leaves, Giovanelli and Stumpf (4) also reported the necessity of Mn for the decarboxylation of malonate. Studies by Hayaishi (6), Nakada (11, 12), and Wolfe et al (18), however, showed that Mg was the metal required in the decarboxylation, and that Mn could only partially substitute for Mg.

TABLE VI

EFFECT OF VARIOUS METALS ON MALONATE DECARBOXYLATION RATE

METAL	μl CO_2/HR/VESSEL
$MnS_4 \cdot H_2O$	144
$MgSO_4 \cdot 7\ H_2O$	0
$FeSO_4 \cdot 7\ H_2O$	8
$ZnSO_4 \cdot 7\ H_2O$	43
No metal	25

Metal concentration = 96 ppm.
Substrate = 60 μM malonate.
Endogenous rate = O.

The necessity of ATP and CoA in the decarboxylation of malonate suggested that CoA-activated malonate might be involved in the reaction. Therefore, hydroxylamine was used as a trapping agent to detect the formation of such an intermediate. One hundred twenty μM of 2-C^{14} malonate were incubated with a particulate preparation, pH 7.0, in nitrogen atmosphere for 3 hours. Cofactors added were ATP, CoA, GSH, and 200 μM $NH_2OH \cdot HCL$. Manganese was omitted to facilitate the isolation of the malonyl-hydroxamate derivative(s). Following the 3-hour incubation, the reaction mixture was centrifuged at 30,000 × G and the supernatant lyophilized and chromatographed. The chromatography data (table VII)

TABLE VII

R_f VALUES OF HYDROXAMATE DERIVATIVE ISOLATED BY ONE DIMENSION PAPER CHROMATOGRAPHY *

HYDROXAMATE DERIVATIVE	PUBLISHED R_f (12)	OBSERVED R_f
Acetyl hydroxamate	0.51-0.54	0.47
Malonyl monohydroxamate	0.35-0.38	...
Malonyl dihydroxamate	0.10-0.15	0.14

* Solvent: water saturated butanol.

indicated that 2 hydroxamates were present. The R_f values corresponded very closely with published values for acetyl hydroxamate and malonyldihydroxamate (12). The hydroxamate spots were counted under a gas flow counter and found to possess substantial radioactivity. The existence of a malonyl hydroxamate indicated the metabolism of malonate proceeded by way of malonyl-CoA. Other investigators working with fungi and mammalian preparations have shown the existence of malonyl-hydroxamate derivatives by similar techniques (6, 12, 18).

From the above experiments it is quite clear that malonate may serve as a substrate for Krebs cycle oxidation in bush bean leaves. Since acetate was a product of malonate metabolism, malonate may also serve as a substrate for other reactions. Recent work by Brady (2) indicated that malonyl CoA was in-

volved in fat metabolism by condensation with an aldehyde to form long chain fatty acids. This discovery appears to link malonate to a very important metabolic pathway, namely, fatty acid synthesis.

Summary

Data presented indicated that malonate was a major constituent of the organic acids of bush bean leaves. Malonate was shown to be readily metabolized by the particulate fraction. The formation of labeled acetate from 2-C^{14} malonate under anaerobic conditions indicated that acetate was a participant in a pathway of malonate metabolism. The occurrence of labeled Krebs cycle acids suggested the condensation of labeled acetate with OAA to form labeled citrate. The cofactors required for the decarboxylation of malonate were found to be CoA, ATP, GSH, and Mn. The identification of malonyldihydroxamate indicated the metabolism of malonate proceeded by way of malonyl CoA.

Thus it would appear that the pathway of malonate metabolism in bush bean leaves was similar to the pathway previously reported for fungi and mammals, one possible difference being the metal required.

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ENZYMATIC DEGRADATION OF ADENOSINE TRIPHOSPHATE TO ADENINE BY CABBAGE LEAF PREPARATIONS[1]

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Apyrases, enzymes which hydrolyze ATP (adenosine triphosphate to form AMP (adenylic acid) and orthophosphate, have been described in potato (11) and citrus fruits (1). A phosphatase which will specifically dephosphorylate AMP has been found in potato (18); however, non-specific phosphatases will also dephosphorylate this compound (2). Enzymes specifically dephosphorylating yeast adenylic acid (A3P) are known in rye grass, wheat leaves, and barley (31). Nucleosidases which will hydrolyze adenosine to adenine have been demonstrated in potato (7), in wheat (29), and in soy bean leaves (22). This paper describes the enzyme complex in the cytoplasm of cabbage leaves which is capable of the breakdown of ATP to adenine. The route of breakdown appears to proceed to AMP by an apyrase reaction, followed by a dephosphorylation and hydrolysis to yield adenine. Two apyrases are present in the cytoplasm. An apyrase associated with the washed cytoplasmic particles has been found that is distinct from the apyrases of the soluble fraction.

MATERIALS AND METHODS

PREPARATION OF ENZYMES: Cabbage heads were purchased at local markets. The leaves were separated and washed with distilled water and stored in cellophane bags at 4° C. The leaves were used within 7 days of purchase. All of the following operations were carried out in the cold. The large midribs were removed and the leaf blades were cut into small pieces and blended with an equal volume of cold 0.5 M sucrose in a Waring blendor for 30 to 60 seconds with a voltage regulator control set at 60 to 80 volts. The blendor was rocked during this period to get all the leaf material into the blades. The homogenate was strained through cheesecloth and then centrifuged for 5 minutes at 1000 × G. The sediment was discarded and the suspension centrifuged for 20 minutes at 23,000 × G. The cytoplasmic supernatant solution was decanted and stored at −10° C. The particulate sediment was resuspended in 0.5 M sucrose and centrifuged again. The washed particulate material (WP) was resuspended in 0.5 M sucrose and used as an enzyme source. In most experiments it was recentrifuged and resuspended again before use. This material is called 2WP.

The frozen cytoplasmic solutions of several experiments, which in some instances had been stored for 90 days, were thawed and brought to 75 % saturation with solid $(NH_4)_2SO_4$ in the cold. The precipitate was recovered by centrifuging and dissolved in cold, distilled water. Insoluble material was removed by centrifugation. The solution was dialyzed overnight against several changes of cold distilled water. The dialyzed solution was clarified by centrifugation and the clear supernatant was lyophilized and stored at −10° C. A distilled-water suspension of the lyophilized cytoplasm (LC), usually 0.5 %, was made. The solution obtained after centrifugation was used as the enzyme source. Protein concentrations were determined spectrophotometrically (12) in the case of LC solutions and by micro-Kjeldahl analysis for N when WP and 2WP were used.

STANDARD ASSAY PROCEDURE: Acetate buffer was used at acid pH, collidine at neutral pH, and tris-(hydroxymethyl)-aminomethane at alkaline pH. Sixty micromoles of buffer of the desired pH was customarily used, with 5 micromoles of substrate, enzyme, and distilled water to a final volume of 1 ml. After incubating the reaction mixture at room temperature (with some shaking) for the desired time, the reaction was stopped by addition of 0.2 ml of 1.5 M perchloric acid. The resulting precipitate was removed by centrifugation. Inorganic phosphate was determined by the method of Fiske and Subbarow (19). Controls in which the LC or WP had been heated in boiling water for 5 minutes before use were frequently run. The amount of phosphate found in these controls was always negligible.

Paper chromatography was carried out by one-dimension paper chromatography with either the solvent system of Cohn and Carter (5) or with butanol : acetic acid : water (25).

CYTOPLASMIC SYSTEM: *Proof of ATP breakdown by an apyrase reaction*: LC was incubated with ATP or ADP (adenosine diphosphate) at pH 6.8 and aliquots of the reaction mixture were examined by paper chromatography after various time intervals. When ATP was the substrate and 5.5 mg. of LC were used, ADP was present after 5 minutes but AMP was absent. After 15 minutes only AMP and adenine could be detected on the chromatogram. With ADP as the substrate both AMP and adenine were present at the end of 5 minutes, and the ADP had completely disappeared. No trace of ATP was ever observed as a product starting with ADP. The above results would seem to exclude the possibility that a simple ATPase breakdown to ADP followed by an adenylic kinase reaction is occurring. If an adenylic kinase is present, then its reaction rate must be very slow compared to the ATPase to yield the above results.

Pathway from AMP to adenine: The breakdown of AMP to adenine could proceed either by a dephosphorylation to adenosine followed by a hydrolysis (7, 22, 29) or phosphorolysis (13) to adenine or by a

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[2] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

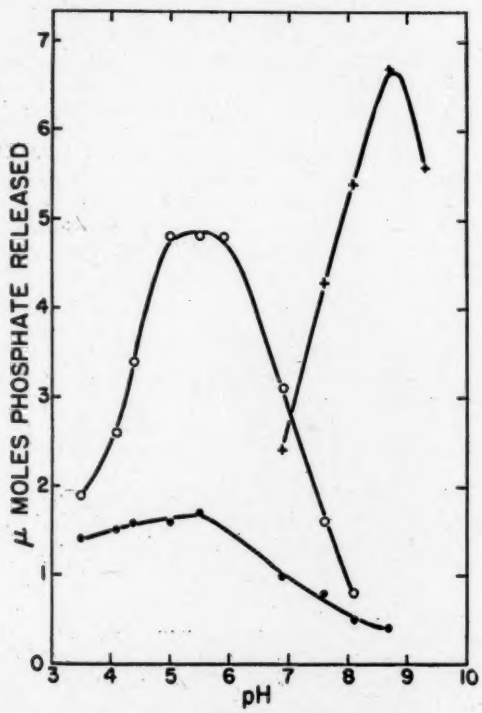
direct hydrolysis (9) or phosphorolysis (16) at the purine-ribose linkage to yield adenine and a phosphorylated ribose. There are several lines of evidence which indicate that the route from AMP to adenine is by way of a dephosphorylation to adenosine and then hydrolysis to adenine.

First, even though adenosine has never been found on any chromatogram in which adenylic nucleotides were converted to adenine, if LC is added to an adenosine solution, adenine is formed rapidly. This conversion can occur in the absence of phosphate. Thus a very active hydrolytic adenosine ribosidase is present. Studies of phosphatase activity, which will be discussed later, show that LC is capable of dephosphorylating AMP, A3P, and ribose-5-phosphate (R5P). Three milligrams of LC will dephosphorylate between 25 and 30 % of the added substrate in 30 minutes at neutral pH. Paper chromatography showed that adenosine was converted more rapidly than AMP to adenine at the same concentration. Thus if the conversion of adenosine to adenine is much more rapid than the breakdown of AMP to adenosine, as soon as adenosine is formed it would be converted to adenine, and a sufficient concentration of adenosine necessary for visualization on a paper chromatogram would never be accumulated. Roberts (29) has shown with wheat leaf preparations that incubation with AMP forms small amounts of adenosine compared to adenine, and concludes that the pathway from AMP to adenine in this tissue is via adenosine.

Second, at pH 8.1 AMP is not dephosphorylated, and concomitantly no adenosine or adenine is found. However, adenosine is hydrolyzed at this pH. It would seem to imply that the phosphate group must be removed before further hydrolysis can occur.

Third, no R5P has been found as a product of the reaction either by paper chromatography, or by use of a linked enzyme system composed of transketolase, phosphoribose isomerase, phosphotriose isomerase, and α-glycerophosphate dehydrogenase (8).

Specificity of the nucleosidase: Incubation of LC with different nucleosides, followed by paper chromatography revealed that only adenosine was hydrolyzed to adenine by cabbage LC. Uridine, inosine, xanthosine, and guanosine were untouched. The reaction was run in phosphate buffer to include a test for the presence of a nucleoside phosphorylase (13) also. These results are similar to those found with wheat leaves (29) in that only adenosine was hydrolyzed.



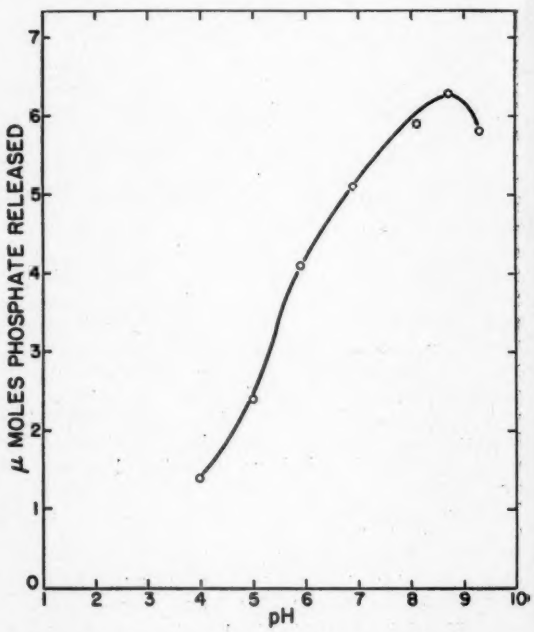


FIG. 1 (*left*). The amount of inorganic phosphate produced as a function of the pH with LC. ○ = ATP as substrate without Mg, x = ATP as substrate with 10 μM Mg^{++} present, ● = AMP as substrate, 1 to 1.2 mg LC were the enzyme source. The reaction time was for 15 minutes at room temperature with ATP as the substrate and for 30 minutes with AMP.

FIG. 2 (*right*). The amount of inorganic phosphate produced as a function of the pH with WP. Incubated at room temperature for 30 minutes with WP containing 1 mg N.

Soy bean leaves possess systems capable of hydrolyzing both pyrimidine and purine nucleosides (22).

pH optima of apyrase and AMPase activity: Two apyrases have been found, one with an acid pH optimum requiring no metal ion, and the other an alkaline pH optimum and needing Mg^{++} for activity. Figure 1 shows the curves of activity against pH for the apyrases. Each curve is a different experiment. Two inorganic pyrophosphatases (P-Pase) were also present with similar pH optima and metal requirements to the apyrases.

Certain differences between the acid apyrase and P-Pase in their response to pH were observed. On the acid side the pH optimum for the P-Pase was approximately 4.6 and for the apyrase was approximately 5.0. The breakdown of ATP had a broader peak than the curve for P-P hydrolysis. Above neutrality there was a very sharp drop in activity. Addition of Mg^{++} activated the breakdown of both P-P and ATP by enzymes having pH optima at 8.7.

The dephosphorylation of AMP reached an optimum in the region from pH 5.0 to 5.5. Above neutrality the activity dropped to low levels and could not be restored by Mg^{++}.

TABLE I

EFFECT OF VARIOUS METALS ON CYTOPLASMIC APYRASE ACTIVITY AT DIFFERENT pH

pH	PROTEIN	ACTIVITY			
		−METAL	$+Mg^{++}$	$+Mn^{++}$	$+Ca^{++}$
	mg				
5.0	1.2	5.6	4.9	3.7	5.6
6.9	1.8	6.0	3.5	1.7	3.2
8.1	1.2	1.0	4.6	0.7	1.1

* Micromoles of orthophosphate produced in 15 minutes.

Metal effects on apyrase: Table I shows that only Mg^{++} activated the apyrase in the alkaline region. An interesting observation is the inhibition of apyrase activity by Mg^{++} at neutral pH. Replicate experiments showed that 0.01 M Mg^{++} inhibited the apyrase activity from 38 to 55 % at pH 6.9. P-Pase was not affected under these conditions. Below pH 6.9 there was little inhibition by Mg^{++} or Ca^{++}.

Inhibition studies of apyrase: Apyrase activity was inhibited 90 % by 10^{-3} M molybdate at pH 6.9 or lower. At the acid and alkaline optima for the apyrases, 5×10^{-2} M F^- is very good as an inhibitor. Incubation of the enzyme preparation with EDTA (ethylenediaminetetraacetic acid) for 15 minutes prior to addition of the substrate had no effect on the acid apyrase. At neutral pH the effect of added F^- decreased. Only 60 % inhibition was obtained with the same concentration of F^- which gave 90 % inhibition at the acid and alkaline optima.

Specificity of LC for phosphate and pyrophosphate esters: Table II is a summary of the ability of LC preparations to hydrolyze various phosphate esters

TABLE II

SUBSTRATE SPECIFICITY OF CABBAGE LC

SUBSTRATE	ACTIVITY*	
	pH 5.0	pH 8.7**
ATP***	6.0	7.0
ADP***	4.4	2.8
AMP	1.7	0.4
A3P	3.0	0.3
UTP***	6.4	2.2
Uridylic acid	2.2	0.4
R5P	1.9	0.4
Glucose-6-PO_4	2.3	0.2
Hexosediphosphate	4.9	1.0
Phosphoglycoric acid	2.9	0.5
Na β-glycerophosphate	3.2	0.1
Inositol monophosphate	1.6	0.1
Flavin mononucleotide	2.9	0.4
Na P-P***	8.3	9.0

* Expressed as μM orthophosphate released by 1 mg protein in 30 minutes except as noted.

** Includes 10 μM Mg^{++} in reaction mixture.

*** Incubated for 15 minutes.

to give inorganic phosphate. At pH 5.0 a large number of phosphate esters are hydrolyzed. Pyrophosphate esters such as ATP, ADP, UTP (uridine triphosphate), and P-P are very readily attacked and monophosphate esters are also hydrolyzed. Note that the pyrophosphate compounds were incubated for only half as long as the simple phosphate esters.

Substituted pyrophosphates and phosphodiesters were used as substrates and the presence of cleavage enzymes determined by the appearance of orthophosphate through the action of the acid phosphatase. Table III gives the results. Uridine diphosphoglu-

TABLE III

ABILITY OF CABBAGE LC TO CLEAVE PHOSPHATE DIESTERS AND PYROPHOSPHATE DERIVATIVES

SUBSTRATE**	ACTIVITY*	
	pH 5.0	pH 6.9
Uridine diphosphoglucose	1.4	1.9
DPN	3.5	4.4
DPNH	5.3	6.6
L-β-Glycerophosphorylethanolamine	0.5	0.6
Ribonucleic acid	1.6	0.8

* Micromoles of orthophosphate released in 60 minutes with 2.8 mg protein.

** Five μM in each case except with ribonucleic acid, where 1 mg was used.

cose, DPN DPNH (oxidized and reduced diphosphopyridine nucleotide, respectively), and ribonucleic acid were cleaved at both pH 5.0 and at 6.9. The higher pH was more effective except in the case of the ribonucleic acid. Since pH 5.0 is the optimum for the phosphatase activity this would imply that the cleavage enzymes work much more rapidly at the

higher pH. L-*a*-Glycerophosphorylethanolamine was not cleaved at either pH. The DPNH was not oxidized prior to cleavage. A nucleotide pyrophosphatase similar to that in potato (17) and a ribonuclease seem to be present.

PARTICULATE SYSTEM: *Conversion of ATP to adenine*: WP converted ATP and ADP to AMP very rapidly at neutral pH. The AMP was then slowly hydrolyzed to adenine. Adenosine was not detected on any paper chromatogram. Time experiments with ADP as the substrate indicated as before that adenylic kinase was not involved in this conversion. With ATP as substrate, ADP and AMP are detected after a short time. ATP then disappears, followed by ADP leaving AMP and adenine. Lyophilized WP retained the complete system which could not be extracted by water. Repeated washing of the particles in 0.5 M sucrose during preparation showed that even particles washed 4-times had good apyrase activity. Continued washing appeared to lower the rate of conversion of AMP to adenine. The ability to convert adenosine to adenine also was gradually lost, but adenosine was still not observed as an intermediate from AMP to adenine under these conditions. Figure 2 shows apyrase activity as a function of pH.

Unlike the soluble system, there is an enhancing effect of Mg^{++} at any pH. The optimum is at pH 8.7.

Magnesium ion and effect of inhibitors: Table IV shows the effects of addition of Mg^{++}, F^-, molybdate, and EDTA on the apyrase activity at different

TABLE IV

EFFECT OF Mg^{++}, F^-, MOLYBDATE, AND EDTA ON THE PARTICULATE APYRASE WITH VARYING pH

pH	N	Activity*				
		Control	+10 μM Mg^{++}	+50 μM F^-	+1 μM Molybdate	+5 μM EDTA
	mg					
5.0	0.8	2.5	4.0	0.8	0.6	2.8
6.9	0.8	5.0	6.8	2.2	3.0	3.2
8.7	0.3	5.5	6.2	4.6	3.6	0.5

* Micromoles of orthophosphate released in 30 minutes.

pH values. The particulate enzyme has considerably different properties from either of the soluble apyrases. It is 75 % inhibited at pH 5.0 by F^-, and molybdate, 40 to 50 % inhibited at pH 6.9, but only slightly inhibited at its optimum pH. Mg^{++} stimulates to a significant degree at all pH levels. Pre-incubation of the particles with EDTA does not remove the residual activity at pH 5.0, inhibits 36 % at pH 6.9, but completely inhibits at pH 8.7. This may be related to the requirement that the EDTA be in the ionized form to act as an inhibitor.

Substrate specificity of WP: 2WP had relatively slight activity toward simple phosphate esters at both pH 5.0 and 6.9. If enough time is allotted, however, considerable hydrolysis of AMP will take place. Table V shows that of the simple esters only hexose

TABLE V

SUBSTRATE SPECIFICITY OF 2WP

Substrate	Activity*	
	pH 5.0**	pH 6.9***
ATP	1.8	4.2
UTP	2.0	5.9
AMP	0.4	0.7
R5P	0.5	0.7
Hexosediphosphate	1.2	1.8
Na β-glycerophosphate	0.9	—
Na P-P	2.9	3.7
Inositol monophosphate	0.5	0.4
Phosphoglyceric acid	0.7	1.1

* Micromoles of orthophosphate from 2WP containing 0.6 mg N.
** Incubated 30 minutes at room temperature.
*** Incubated 15 minutes at room temperature.

diphosphate and phosphoglyceric acid are hydrolyzed to any extent at either pH. ATP, UTP, ADP, and P-P were hydrolyzed very readily. UTP appeared to be a better substrate than ATP at either pH.

DISCUSSION

The observation of Naganna et al (24) that cabbage possesses 2 inorganic pyrophosphatases distinguishable by their pH optima and Mg^{++} activation was confirmed. Soluble apyrases have also been demonstrated which are active in combination over a wide range of pH. The cytoplasmic particles of cabbage leaves have been shown to contain apyrase and pyrophosphatase activity. Particulate apyrases have been found in yeast (20), brain (21), and chick embryos (32). Rafter (27) has recently shown that mouse liver particles possess pyrophosphatase activity. Since all isolated enzymatic reactions must eventually be integrated into the physiology of the cell as a whole, one is justified in speculating as to the function these particular enzymes perform in the economy of the cell.

It is difficult to conceive a role for the presence of apyrase in both soluble and insoluble cell fractions at the present time. A role for apyrase in the fermentation of yeast was shown by Meyerhof (20). The function of the apyrase was to provide a phosphate acceptor at the 1,3-diphosphoglyceric acid step. Engelhardt (6) believes that this role of apyrase must be considered in all those cases where adenylic nucleotides are involved as phosphate acceptors and not as an energy source. Racker (26) has made the interesting speculation that the non-specific phosphatase activities of cell-free preparations may be examples of group transfer reactions that have developed into simple hydrolyses. By this reasoning, apyrase could be an enzyme or complex of enzymes that customarily

transfer adenyl or adenosine diphosphoryl groups to some acceptor and transfer them to water under the present experimental conditions because of either lack of acceptor or through an artifact of preparation.

One may conjecture that the function of inorganic pyrophosphatase may be to act in a linked synthetic system to affect the biosynthesis of compounds under unfavorable equilibrium conditions. Many reactions involving ATP as one of the reactants have pyrophosphate as an end-product. The synthesis of DPN (15), and flavin-adenine dinucleotide (30); the activation of acetate (10), sulfate (4, 28), and carbon dioxide (3); the formation of intermediates in carbohydrate (23) and phospholipid (14) synthesis are all examples in which P-P is an end-product. Often the equilibrium is unfavorable for synthesis, and the hydrolysis of the P-P formed could be used to drive the reaction to completion.

Summary

Water extracts of lyophilized ammonium sulfate fractions of the cytoplasm from cabbage leaf homogenates are capable of readily degrading ATP completely to adenine. Washed cytoplasmic particles and suspensions of lyophilized particles can also carry out this degradation. The pathway of breakdown appears to be by means of an apyrase reaction to adenylic acid, followed by a relatively slow dephosphorylation to adenosine followed by a rapid hydrolysis to adenine.

The cytoplasm has 2 apyrases which can be distinguished by pH optima and response to Mg^{++}. One enzyme is an acid apyrase with a pH optimum at 5.0 requiring no added Mg^{++}. The other is an alkaline apyrase with a pH optimum at 8.7 and a requirement for added Mg^{++}. Associated with each apyrase is the ability to split inorganic pyrophosphate. In the case of the acid apyrase, it has been shown that these activities are associated with different enzymes which can be distinguished by differences in pH optima and response to Mg^{++}. The soluble cabbage leaf preparation has also been shown to contain a very active hydrolytic adenosine ribosidase, ribonuclease, and nucleotide pyrophosphatase in addition to an active acid phosphatase.

The possible functions of apyrase and pyrophosphatase in cell metabolism are discussed.

I would like to thank Mr. Lawrence White and Miss Geraldine Secor for carrying out the micro-Kjeldahl analyses.

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GROWTH AND DEVELOPMENT OF ISOLATED PHYCOMYCES SPORANGIOPHORES[1]

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The large sporangiophores of Phycomyces (Order Mucorales) have long been favorite objects for research in several areas of fungus physiology. However, these asexual reproductive structures differ greatly in development from the vegetative mycelium, and none of the information now available on the nutritional requirements and metabolism of entire Phycomyces colonies applies to the sporangiophores themselves.

In order to achieve an understanding of the metabolic processes involved in the growth and tropistic responses of sporangiophores, it seemed imperative to study these structures separately from the mycelium.

The feasibility of removing Phycomyces sporangiophores intact from the mycelium has been mentioned several times in the literature. Laurent (13) determined certain osmotic quantities of isolated sporangiophores. Burgeff (3) excised sporangiophores for his hybridization experiments, and thought that they remained turgid because the basal ends were apparently plugged with cytoplasm. Grehn (8) made a few observations on the growth of isolated Phycomyces sporangiophores as part of his studies on the sporangiophores of several mucoraceous fungi. Isolated immature sporangiophores placed horizontally on malt agar blocks, with their bases and apical portions in air, stopped growing for a few hours due to "wound shock," but then resumed growth by first forming sporangia. The morphology of these specimens was normal, and there was no regeneration of mycelium at the base; their phototropic and geotropic sensitivity was retained. In a parallel experiment with an isolated sporangiophore wedged between agar blocks (basal and apical portions in air) Grehn found that the growth rate was reduced by almost 50 % when compared with that of intact sporangiophores, and that the final length (61 mm at 39 hours after resumption of growth) was less than normal (98 mm in 32 hours). No other data are given. Grehn thought that sporangiophores treated in the manner described must obtain all their nutrients and water through the cell wall, which was somewhat lighter and more transparent at the contact area. He speculated that a lack of water might be an important, although not necessarily the sole cause of the reduction in growth. When isolated sporangiophores were embedded in small gypsum blocks placed on nutrient agar most of them produced branches after 18 to 24 hours.

Isolated sporangiophores were used by Roelofsen (14) in his "iron lung" experiments, and by Johannes (10) in work on vital staining with fluorescent dyes. Other reports in the literature indicate that intact sporangiophores of Pilobolus, a genus related to Phycomyces, can also be removed from the mycelium. For instance, Bünning (2) studied the elastic extension of the cell wall of isolated sporangiophores of *Pilobolus kleinii*.

However, in none of this work, apart from Grehn's limited observations, were the isolated sporangiophores actually grown, nor were they maintained for any length of time.

In preliminary experiments the author (9) found that isolated Phycomyces sporangiophores could be grown for a considerable time on water or other liquid substrates. This finding, recently confirmed

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by Cohen and Delbrück (5), suggested a line of approach which would allow the use of mass cultures of isolated sporangiophores for studies on various aspects of their physiology. The technique, and first results of this research are described below.

Materials and Methods

A + strain of *Phycomyces blakesleeanus* (G-5) has been used in all experiments. Spores and mycelium (sometimes only spores) were inoculated at one site near the wall on sterile potato-dextrose-agar medium in 5 cm deep crystallizing dishes closed with Petri dish covers. The medium contains the following ingredients in 1 liter of distilled water: decoction from 400 g peeled potatoes cut into small pieces, boiled for 1 hour, and strained through several layers of cheesecloth; 20 g dextrose, and 15 g granulated agar (Difco). The cultures were kept under a weak overhead light until young sporangiophores appeared on the mycelium in fairly large numbers (mycelium covering between one half and three fourths of the agar surface).

Culture chambers for the isolated sporangiophores were set up in the following manner: a Petri dish base (10 cm diameter, fig 1, A) supported the lower half of a smaller Petri dish (5.4 cm diameter, fig 1, B), and a slide cut to 5.5 cm in length (fig 1, C) was placed across the middle of the inner dish. An inverted 400 cc beaker with level rim (dye-pot, height: 13 to 15 cm) was used to cover the cultures (fig 1, D). The culture chambers were sterilized by autoclaving, and sterile conditions were maintained in all subsequent steps carried out in a transfer room. A small amount of partly molten petrolatum was smeared along both edges of the slide lying across the inner Petri dish. Pyrex re-distilled water was then added to this dish to a level about 5 to 6 mm below the slide. By means of fine forceps sporangiophores of comparable diameter were removed from the mycelium and held next to a ruler without touching. Measurements were made to the nearest millimeter. After partially lifting the cover of the culture chamber the sporangiophores were gently attached to the vaseline-covered slide edge with the base dipping into the liquid. Tests for sterility were carried out several times by streaking on potato-dextrose agar, but no contamination was observed.

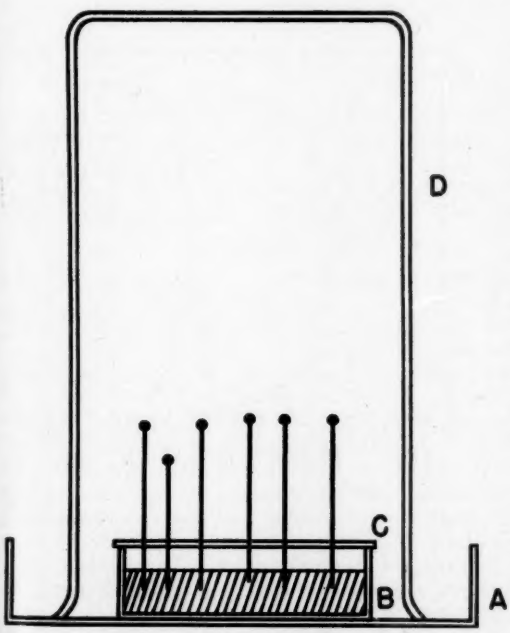

Fig. 1. Culture chamber for isolated sporangiophores. See description in the text.

The cultures were kept at 23± 1° C in a closed box which allowed continuous illumination from above through layers of white paper. A white fluorescent tube was used as the light source for all experiments, the intensity of illumination being very close to 1 ft-c at the level of the culture dishes. For final readings sporangiophores were placed on moist filter paper and measured with the ruler. Lastly, the individuals from each culture (basal halves only if they were long) were placed in the same sequence on slides with lactophenol-cotton blue. Short-term growth measurements were carried out with a horizontal Leitz microscope equipped with a Filar micrometer eyepiece (Bausch and Lomb).

For purposes of comparison growth measurements were also carried out on sporangiophores connected with the mycelium. These were grown in 10 cc beakers filled with potato-dextrose agar to 1.5 cm below the rim. When enough sporangiophores had grown to a height between 1.5 and 2 cm those outside this size range were removed. While this procedure is laborious, and does not prevent initiation of a 2nd crop, the specimens to be measured can still be distinguished after many hours. One or two additional extirpations of 2nd growth sporangiophores were usually carried out in long-term studies. However, after a certain time the cultures can no longer be touched in view of the length of the sporangiophores. Thus, after about 3 days from the start, some of the sporangiophores measured may have actually grown for a shorter time. However, the average final growth will at most be somewhat underestimated.

For short-term studies these cultures were kept in the chambers described above (fig 1), but for long-term growth measurements they were transferred to chambers made of 2 beakers mouth to mouth, or covered by a lucite box.

For dry weight determinations sporangiophores were grown under the standard conditions of light and temperature on potato-dextrose agar in 6 cm Petri dishes covered by tall beakers. Shortly after removal from the mycelium the sporangiophores were placed in small beakers, and dried to constant weight for 12 hours at 80° C.

The designation of stages in sporangiophore de-

velopment used in this work is essentially that of Castle (4); Stage 1 sporangiophores have not yet formed sporangia; Stage 2 is marked by the initiation and enlargement of sporangia, accompanied by cessation of elongation for a few hours; in Stage 3 sporangia are fully formed, but elongation has not yet resumed; Stage 4 marks the resumption of growth and the maturation of spores, and comprises the major growth period of Phycomyces sporangiophores. Castle's subdivision of Stage 4 into 4 a and 4 b depending on the direction of spiraling must be ignored for present purposes. The sporangia are yellow during Stages 2 and 3, and it should be noted that the resumption of elongation in Castle's Stage 4 begins while the sporangia are still yellow (although they turn brown rapidly thereafter). This has been pointed out by Roelofsen (15), and has been observed repeatedly by the present author. In view of the impossibility of distinguishing Stages 2 and 3 from the early Stage 4 by gross observation, Stage 2–3 in the present investigation designates sporangiophores with yellow sporangia, and Stage 4 those with brown to black sporangia.

Results

General Observations: Sporangiophores isolated in Stage 1, and cultured on water, continue growing for many hours and in most instances undergo normal development. This stage is the one best suited for experimental work. Stage 2–3 sporangiophores can also be isolated, but some collapse at the apex immediately after removal from the mycelium, and many others grow abnormally (see below). Mature Stage 4 sporangiophores are difficult to isolate because so many collapse in the region of the growth zone; however, those that have been removed undamaged continue growing. In view of these findings the present work has been largely restricted to sporangiophores isolated in Stage 1.

Phycomyces sporangiophores are devoid of crosswalls, and are not separated by a septum from the main hyphal trunks. Nevertheless, the contents of isolated Stage 1 sporangiophores do not flow out although at times a minute amount of extruded cytoplasm is visible at the torn-off base just after removal from the mycelium. However, these individuals do not collapse, and it is impossible to say whether the material in question comes from the sporangiophores or the hyphae. Preliminary studies with both ordinary and phase contrast microscopes did not yield definite clues to the different behavior of sporangiophores on removal in different stages. The lowermost portions of the sporangiophores in all stages, including early Stage 4, appear to be filled with a cytoplasmic plug of variable length, which delimits the central vacuole (very large in Stage 4).

The point at which growth resumed after isolation can often be detected by a slight constriction of the sporangiophore, which then tapers steadily towards the apex. The distance from the base to the short narrow region has shown good agreement with the original length.

Sporangiophores generally become narrower towards the base, and connect with a relatively wide hypha at a point which is frequently distinguishable on isolated specimens by a slight widening. The large hypha is torn off close to the sporangiophore base, and sometimes also carries short amputated sections of other hyphae. The globular lateral branches which Grehn (8) designated as storage vesicles in Stage 1 are very rarely encountered at the base of isolated sporangiophores although they are present abundantly on the surface hyphae of the mycelium. It should be noted that isolated sporangiophores grew well without even remnants of any branch hyphae at the base.

In water the majority of isolated sporangiophores were not found to regenerate mycelium visible to the naked eye. The possibility that very short hyphal branches are sometimes regenerated directly at the base cannot be excluded since it was hard to observe the base carefully during preparation of the cultures. The variability in those sporangiophores which were observed with the microscope just after isolation precludes any generalization about their initial condition.

Isolated sporangiophores did not change the pH of distilled water significantly. The average pH in 29 cultures decreased from 5.84 to 5.68 after 64 hours of growth with 20 or more sporangiophores per culture.

Early Development of Isolated Sporangiophores on Water: Observations on sporangium formation in 293 sporangiophores were made up to 20 hours after isolation in Stage 1. Figure 2 gives the percentages of the total number of specimens found in each stage of development at the indicated periods

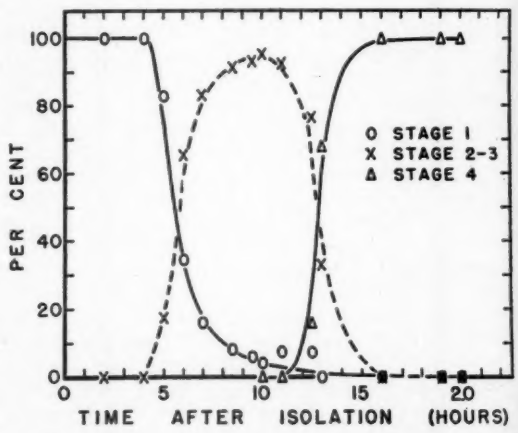

Fig. 2. Early development of sporangiophores isolated in Stage 1 (initial length: 1 to 2 cm), and grown on water at 23 ± 1° C. The curves give the percentages (ordinate) of the total number of individuals in each stage of development at successive time intervals (hours) after isolation (abscissa). ○, Stage 1 (no sporangia); X, Stage 2–3 (yellow sporangia); △, Stage 4 (dark sporangia).

after isolation. All except the 16 hour and some of the 13 hour values are from 3 series of cultures, the cultures in 2 series being observed only once at different times, and those in the 3rd several times. Most of the sporangiophores grew longer than the time indicated in the figure, thus allowing detection of abnormalities. Despite some variability, the data show that sporangium initiation, which began between 4 and 5 hours, was 90 % complete by 8 to 8.5 hours (fig 2, stippled curve), while sporangium maturation (darkening) began between 11 and 12 hours, and was completed between 14 and 16 hours after isolation. Recalling that sporangia are still yellow at the beginning of the final period of elongation, it is safe to assume that Stage 2–3 is essentially completed by, at most, 13 hours.

In contrast to this behavior of isolated sporangiophores it is clear that the development of attached Stage 1 sporangiophores of 1.5 to 2 cm initial length varied much more between series of cultures started on different days. Sporangia were initiated in some cultures before 3 hours, in others only at 7 hours. Yellow sporangia persisted longer in cultures of attached than of isolated sporangiophores. For instance, at 17 to 18 hours, 14 % of 50 attached sporangiophores still had yellow sporangia while all isolated specimens were dark at 16 hours (fig 2). A few attached sporangiophores with yellow sporangia were observed even up to 22 hours. Maturation (darkening) of sporangia was also quite variable in attached 1.5 to 2 cm sporangiophores, and there was no such sharp overall separation of stages as in figure 2. Thus the isolated sporangiophores show considerably greater synchronization than those attached to the mycelium.

TIME COURSE OF GROWTH OF ISOLATED AND ATTACHED SPORANGIOPHORES: Observations with the microscope showed that only 3 of 15 Stage 1 sporangiophores were growing between 12 minutes and 1 hour after isolation. The rate was very low, 1 to 4 μ/min. Between 1.5 and 3 hours, 7 of the 15 were growing at a mean rate of 7 μ/min. After 20 to 30 hours these 15 specimens, now in Stage 4, elongated at a mean rate of 40 μ/min. A total of 980 measurements of the increase in length of sporangiophores at different periods after isolation yielded the growth curve drawn as a solid line in figure 3. The overall means for each time interval are shown as crosses, and the means for individual runs as dots. For comparison a growth curve for Stage 1 sporangiophores connected with the mycelium was also obtained. Three hundred and sixty measurements gave the stippled curve in figure 3, with large circles indicating overall means, and small circles the means of individual runs. The initial length of isolated sporangiophores was 1 to 2 cm, and of the normal sporangiophores 1.5 to 2 cm (1.8 cm is taken as the average).

The shape of the curves around 10 hours reflects the development of sporangiophores described in the preceding section. Apart from the growth stoppage immediately following isolation, the average growth of excised sporangiophores is very low up to 10 hours because most of them are in Stage 2–3, and have ceased elongating for some time. The steep increase in growth rate between 10 and 13 hours reflects the beginning of the 2nd stage of elongation (Stage 4) in a high proportion of the individuals. This was also suggested by the data in figure 2.

In contrast, the average growth curve for sporangiophores connected with the mycelium rises more steeply from the origin, since some sporangiophores continued growing for more than 1 cm in Stage 1 before initiating sporangia. It is also less steep than the curve for isolated sporangiophores up to about 16 hours because, on the average, Stage 2–3 persisted longer in intact cultures.

The maximum average growth rate of isolated sporangiophores falls approximately between 13 and 30 hours, and is the same as that for normal specimens (42 μ/min., calculated from curve). Figure 3 shows that the elongation of isolated sporangiophores begins to decrease at 30 to 40 hours. The overall means of the two groups at 40 hours are significantly different (t-test, $P<0.001$). Moreover, the isolated sporangiophores stop growing 60 to 80 hours after isolation, while those on the mycelium continue growing much longer, and thus attain a greater average length.

Short-term growth measurements with the microscope were also made on isolated sporangiophores at different times after isolation. The means of these measurements are summarized in table I, and follow the same trend as the data from mass cultures in figure 3. By 64 to 67 hours 9 of 16 sporangiophores had stopped growing.

TABLE I

AVERAGE GROWTH RATE OF SPORANGIOPHORES ON WATER AT DIFFERENT INTERVALS AFTER ISOLATION.*

EXPERIMENT	NUMBER OF SPORANGIOPHORES**	TIME AFTER ISOLATION IN HOURS†			
		20-23.5	40-43	50-51.5	64-67
		μ/min			
1	6	28.8			
2	7	42.3			
3	7	47.6	4.5		0.4
4	9		5.2	2.2	0.9
	Mean††	*40.1*	*4.9*	*2.2*	*0.7*

* Readings with the microscope for 5 minutes (some for 10 to 25 min).

** Initial length: 1 to 2 cm, except 2 of 2.5 cm each in Experiment 3.

† The time interval at the head of each column indicates the longest period between the first and last readings.

†† Calculated from pooled individual measurements.

The curve for isolated sporangiophores in figure 3 is constructed around overall means, and seems to represent a family of curves of similar shape. The dispersion is greatest where the curve decreases in slope (40 to 100 hours), and the overall means would lead one to expect little further growth after about 50 hours. Since this part of the curve was of particular interest the variation was studied by obtaining the average growth of individual cultures in 5 separate

series, each culture being measured at a given time between 40 and 100 hours. The results for 4 series are plotted in the inset of figure 3. A 5th series gave a curve essentially the same as curve 3. The means in curve 4 vary the most, the 90 hour mean being 0.8 cm less than that for 80 hours, but even this discrepancy is small compared to the spread around the overall curve. The data for the other individual series show reasonable internal consistency. Thus there is a family of curves each of which resembles approximately the curve drawn through the overall means. Each of the individual culture series illustrates the fact that growth virtually ceases after about 60 hours. What causes the variation between the means, for the same period after isolation, remains unexplained. Variation in the medium on which sporangiophores grow before isolation can hardly be a significant factor by itself. For instance, the sporangiophores of curve

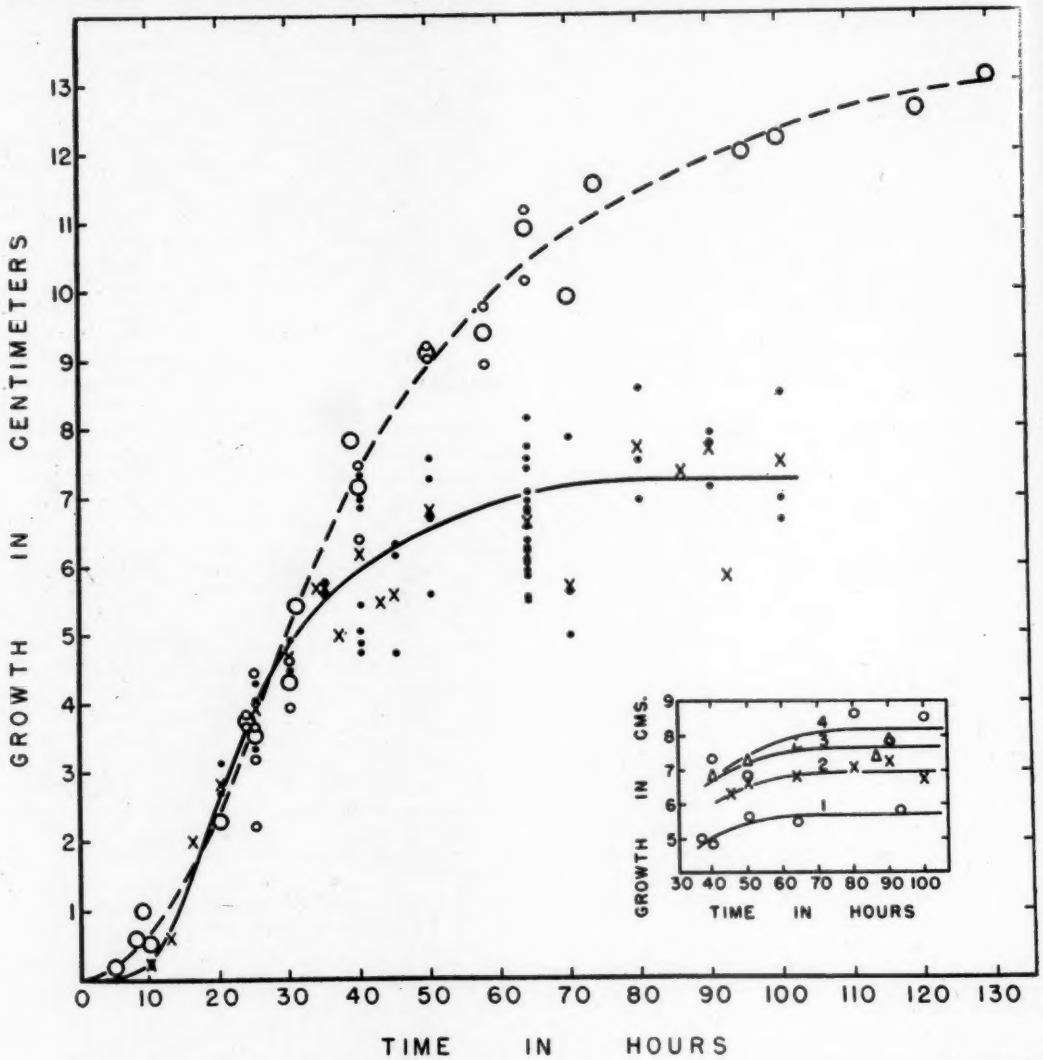

FIG. 3. Time course of growth of sporangiophores at 23 ± 1° C. Solid curve: growth on water of sporangiophores isolated in Stage 1 (initial length: 1 to 2 cm). X, overall means; ●, means of individual cultures. Stippled curve: growth of sporangiophores connected with the mycelium and initially in Stage 1 (initial length: 1.5 to 2 cm). ○, overall means; O, means of individual cultures.

Inset: growth of isolated sporangiophores in 4 series of cultures, each series started on a different day. Abscissa: time in hours after isolation, or after start of readings for sporangiophores on the mycelium. Ordinate: growth in cm.

2 and 4 (fig 3, inset) were grown on the same medium, and were started within 5 days of each other.

In order to test whether the maximum growth of isolated sporangiophores is influenced by light one group of specimens was grown in the dark and another under the standard conditions of constant illumination. All sporangiophores were obtained from the same cultures, and measured 1.2 to 2 cm at isolation. After 80 hours the average growth of 19 sporangiophores in the dark was 5.7 cm, and that of 20 sporangiophores in the light 6.1 cm. Thus the growth of isolated sporangiophores is not influenced by the light used in the present work.

It should be added that any sporangiophores showing indications of damage (see below) were excluded from the above calculations.

GROWTH OF ISOLATED SPORANGIOPHORES IN RELATION TO THEIR INITIAL LENGTH AND DRY WEIGHT: All the above experiments were carried out with Stage 1 sporangiophores of 1 to 2 cm initial length, and the question arose whether growth is influenced by differences in initial length. Table II gives the average growth after 40, 64 and 80 to 100 hours of sporangiophores isolated in Stage 1 and measuring between 1 and 4 cm. These data from many experiments, and for a wide range of initial lengths, show that the maximum growth was attained at close to 64 hours since there was very little or no growth after that period. This agrees with the results presented above for 1 to 2 cm sporangiophores (fig 3). The average maximum growth of 1 to 1.1 cm sporangiophores was 0.4 to 0.6 cm less than that of 1.2 to 4 cm specimens. Within the latter wide range there were no differences in growth after 64 to 100 hours, apart from some variation in the 80 to 100 hour means for 1.9 and 2 cm which include only few measurements.

TABLE II

GROWTH OF ISOLATED STAGE 1 SPORANGIOPHORES OF DIFFERENT INITIAL LENGTH AND OF SPORANGIOPHORES ISOLATED IN LATER STAGES *

	INITIAL LENGTH CM	TIME AFTER ISOLATION 40 HRS	64 HRS	80-100 HRS
Stage 1	1.0	5.7 (21)	6.4 (74)	6.3 (15)
	1.1	5.7 (19)	6.3 (54)	6.4 (19)
	*Means***	*5.7 (40)*	*6.4 (128)*	*6.4 (34)*
	1.2	6.2 (24)	6.8 (89)	7.1 (22)
	1.3	6.5 (29)	6.8 (85)	6.9 (18)
	1.5	6.6 (25)	7.0 (76)	7.1 (27)
	1.7	6.2 (12)	6.7 (53)	6.8 (25)
	1.9	6.6 (11)	7.0 (34)	6.6 (10)
	2.0	6.3 (7)	7.1 (39)	7.4 (11)
	*Means***	*6.4 (108)*	*6.9 (376)*	*7.0 (113)*
	2.1–2.9	5.6 (42)	6.9 (59)	6.8 (36)
	3.0–4.0	5.4 (9)	6.8 (24)	7.1 (20)
	*Means***	*5.6 (51)*	*6.8 (83)*	*6.9 (56)*
Stage 2–3	1.3–2.0	5.3 (15)	6.0 (8)***	...
	2.1–3.4	4.4 (5)	...	5.6 (26)
Stage 4	1.7–3.6	4.0 (9)	...	4.6 (4)

* Substrate: water.
** Calculated from pooled individual measurements. Number of individuals in parentheses.
*** 70 hours.

After 40 hours the average growth was 0.7 cm less in 1 to 1.1 cm than in 1.2 to 2 cm sporangiophores, but the 2.1 to 4 cm specimens also grew less in 40 hours than those in the intermediate range. This suggests some differences in the early growth phase of sporangiophores longer than 1.1 cm even though they give the same maximum growth.

It could be expected that sporangiophores isolated in Stage 2–3 would grow better than those of the same length isolated in Stage 1 since they have had more time in which to accumulate materials from the mycelium (see below, table III). However, table II

TABLE III

AVERAGE DRY WEIGHTS OF SPORANGIOPHORES OF DIFFERENT LENGTH AND STAGE OF DEVELOPMENT GROWN ON POTATO-DEXTROSE AGAR

INITIAL LENGTH	STAGE 1 MEANS OF SEPARATE EXPERIMENTS 1	2	3	OVERALL MEANS	STAGE 2–3 MEANS OF SEPARATE EXPERIMENTS 1	2	3	OVERALL MEANS	STAGE 4 MEANS OF SEPARATE EXPERIMENTS 1	2	3	4	OVERALL MEANS
cm					*micrograms*								
1.0-1.1	44	...	40	42 (80)									
1.6-1.8	73	69	69	71 (65)	...	...	91	91 (25)					
1.9-2.1	91	85	86	87 (72)	...	...	105	105 (23)					
2.5-2.9	128	113	103	111 (58)	123	119	124	122 (38)	...	...	...	104	104 (11)
3.0-3.9	153	...	130	135 (47)	...	...	135	135 (18)	...	...	118	114	116 (41)
4.0-4.9			164	164 (20)			172	172 (25)					
4.5-5.5									142	145	122	...	132 (68)
7.0-7.9									...	...	128	132	130 (17)
8.0-8.9									...	159*	127	144	134 (14)
9.0-9.9									...	...	156	123	142 (26)
10.0-10.9									...	178*	211	...	206 (7)
11.0-12.9									...	...	166	...	166 (9)

Weights in micrograms per sporangiophore averaged for separate and pooled experiments. The number of sporangiophores averaged for the overall means is given in parentheses. A . . . indicates that no measurements were taken, and a blank signifies that the number of sporangiophores in that class was insufficient, or that none were available.

* Only 1 sporangiophore was weighed.

shows that the average growth of Stage 2–3 was consistently less than that of Stage 1 sporangiophores of any initial length. A few measurements on sporangiophores isolated in Stage 4 showed less growth still (table II).

The absence of correlation between growth and initial length suggests that the elongation of sporangiophores isolated in Stage 1, and probably in later stages, is not limited primarily by the amounts of major nutrients present at isolation. Considering the abundance of cytoplasm in Stage 1 sporangiophores it seems probable that those of different length would contain different amounts of major nutrients. Evidence for this view was obtained by determining the dry weights of sporangiophores of different length and stage of development. Table III gives the average dry weights in micrograms per sporangiophore for individual runs, and also the overall means. Four series of experiments were carried out, each with sporangiophores derived from 2 cultures. The pronounced dry weight increase in Stage 1 is almost directly proportional to the increase in length, a doubling in length resulting in a doubling in weight. This fits in well with the above suggestion.

The weight of sporangiophores with yellow sporangia (Stage 2–3) was slightly greater in nearly every instance than the weight of Stage 1 specimens of the same length and from the same experiment. The data for Stage 4 sporangiophores are difficult to interpret since their length and weight in Stage 2–3 is unknown. However, sporangiophores which attain more than 4 cm in Stages 1 to 3 are rare under the present conditions, and were obtained in sufficient numbers only in experiment 3. Generally, sporangia are formed on sporangiophores of more than 2 and less than 4 cm, which will weigh approximately 100 to 135 μg in Stage 2–3, with an upper limit of about 150 μg. Most Stage 4 sporangiophores measuring more than 4.5 cm will have these characteristics in Stage 2–3. From 4.5 to 10 cm the individual mean weights of Stage 4 specimens overlap the above-mentioned range for Stage 2–3 (2.5 to 4 cm), and there seems to be no increase in weight up to 10 cm. Beyond that an increase in weight was observed. Sporangiophores longer than about 10 cm are difficult to handle without loss of part of the sporangium. Hence fewer values for intact sporangiophores were available in that range, and no weighings were made with individuals longer than 13 cm.

An experiment was carried out to determine the dry weight of sporangiophores isolated in Stage 1 and grown on water. The initial length was 1.6 to 2 cm, and the number of normally growing sporangiophores was 8. Sporangiophores of the same length (1.6 to 2.1 cm), and from the same cultures gave an average initial dry weight of 82 μg. After 64 to 67 hours growth on water the final average weight of the isolated sporangiophores was 71 μg, which indicates a slight loss in weight. The final length of these isolated sporangiophores was 8 to 9 cm, and their final dry weight is thus only about half of that of Stage 4 specimens of the same length which remained attached to the mycelium (table III).

Branching and the Effect of Injury: The data presented above are for sporangiophores which continued growing with normal morphology after isolation. However, in most cultures a few individuals produced branches. Of 49 cultures examined between 40 and 100 hours, 41 included branched sporangiophores. In isolated sporangiophores branching could be observed as early as 10 hours, sometimes even earlier. The type of branching is variable. In the majority of cases 1 to 2, or rarely 3 to 4, branches arose close to the original apex, which had stopped growing and often could be stained deeply with cotton blue. Sometimes a single branch arose so close to the apex that the sporangiophore appeared at first sight unbranched, and only slightly bent. In order to eliminate such instances all sporangiophores have been checked with the microscope. An interesting phenomenon is the production of branches from old portions of sporangiophores, far below the original apex, since it suggests that the cell wall can undergo reversible changes. Branches can originate as little as 2 mm above the base, usually appearing at, or just below, a damaged region (see below). Branching can occur simultaneously at the apex and near the base. Sporangia were generally formed at the ends of apical branches, and frequently on lateral branches. Occasionally the original apex also produced a sporangium.

Grehn (8) described various types of symmetrical branching from the apex of sporangiophores embedded in gypsum. He also observed irregular branching, and thought that it was caused by injury to the apex, possibly due merely to contact.

The branching frequency found in the present work does not present any obstacle to the use of the method. Of 956 sporangiophores isolated in Stage 1 (1 to 2 cm), 160 (16.7 %) branched after 40 to 100 hours. However, of 74 sporangiophores isolated in Stage 2–3, 31 (41.9 %) branched. Not enough data are available to make similar counts on sporangiophores isolated in Stage 4, but the formation of branches on the growth zone has been observed.

In 80 (8.4 %) of the 956 sporangiophores isolated in Stage 1 microscopic observation showed clear evidence of internal damage without branching. In the lower portions of these specimens some or all of the following abnormalities could be observed: constriction of the cell wall or cell contents to a varying extent, generally deep staining with cotton blue, and, in some instances, distinct irregularities in the cell wall. Often, cytoplasm of marked abrnormal appearance filled the injured section of the sporangiophores. These effects resemble those due to other types of injury on Phycomyces sporangiophores, studied in detail by Kirchheimer (11).

Many of the branched sporangiophores were also damaged in the manner described above. This is true for almost all cases of lateral branching, but individuals branched only at the apex often showed no

internal injury. Injured, but unbranched, sporangiophores grew much less than those without internal damage. The average growth of 67 damaged sporangiophores after 50 to 100 hours was 3.0 cm, and 66 % fell between 0.3 and 3.0 cm. In a total of almost 700 measurements, 85 % of all specimens which grew 3 cm or less were those which had been damaged. Despite these findings it is true that occasionally greatly reduced growth occurred without any visible evidence of damage.

The only reasonable explanation for the internal damage observed is excessive pressure during handling. This view is supported by the fact that the injury was always encountered in the lower parts of the sporangiophores where they were gripped with the forceps. Other possible causes of abnormalities are injury at the point of separation from the mycelium, failure to immerse the base, and contact between the apical growth zone and the petrolatum at the edge of the supporting slide.

Some injury at the sporangiophore base must always be present, and its effects could not be tested directly. The initial growth stoppage generally observed after isolation might be ascribed to a temporary decrease in the internal pressure due to injury at the base.

The effect of pressure due to handling with forceps was tested by applying strong pressure at the time of isolation in 4 series of sporangiophores grown for 64 hours. The results are shown in table IV. Apart from the data on branching and injury, the important comparison to be made is between the average growth of the unbranched damaged plants in the upper half of the table and that of the undamaged plants in the lower half. It is evident that the treatment increased the frequency of internal damage in spite of the fact that some of the treated sporangiophores escaped detectable injury. However, there was no clear-cut effect of strong pressure on the branching incidence. For instance, the large increase in damaged sporangiophores in experiment 1 b occurred in the absence of any branching. All branched specimens in the treated series and two-thirds of the branched controls showed evidence of internal damage. It might be recalled here that Köhler (12) briefly reported the production of lateral "hyphae" when slight pressure was exerted on Phycomyces sporangiophores.

TABLE IV

EFFECT OF STRONG PRESSURE ON GROWTH AND BRANCHING OF SPORANGIOPHORES OF 1 TO 2 CM INITIAL LENGTH ISOLATED IN STAGE 1 AND GROWN FOR 64 HOURS ON WATER

	Experiments *			Overall
	1 a	1 b	2	3 means **
Strong pressure				
Branched, %	47.4(9)	0	29.2(7)	50.0(9)	32.5
Unbranched				
Damaged, %	42.1(8)	87.5(14)	62.5(15)	11.1(2)	
Average growth, cm	3.9	2.8	3.6	2.4	3.3
Undamaged, %	10.5(2)	12.5(2)	8.3(2)	38.9(7)	
Normal handling				
Branched, %	15.8(3)	0	31.1(14)	31.6(6)	22.8
Unbranched				
Damaged, %	5.3(1)	16.7(3)	40.0(18)	0	
Undamaged, %	78.9(15)	83.3(15)	28.9(13)	68.4(13)	
Average growth, cm	6.9	6.6	6.7	7.1	6.8

All frequency data as percent of total in each experiment and type of treatment, with number of individuals in parentheses.

* Experiments with the same number were run simultaneously.

** Overall means calculated from pooled individual measurements.

TABLE V

EFFECTS OF WATER DEFICIENCY ON GROWTH AND BRANCHING OF SPORANGIOPHORES OF 1 TO 2 CM INITIAL LENGTH ISOLATED IN STAGE 1 AND GROWN FOR 64 HOURS *

Experiment No.	Sporangiophore base in air **		Control base in water	
	Branched, %	Unbranched, average growth cm	Branched, %	Unbranched, average growth cm
1	50.0 (5)	3.7 (5)	0	7.5 (39)
2	9.1 (1)	0.7 (10)	13.5 (5)	6.1 (32)
3	57.9 (11)	1.4 (8)	37.9 (11)	6.4 (18)
Means†	*42.5 (17)*	*1.6 (23)*	*15.2 (16)*	*6.8 (89)*

All frequency data as percent of total in each experiment, with number of individuals in parentheses.

* All sporangiophores without visible internal injury.

** Water in Petri dish.

† Calculated from pooled individual measurements.

In order to test the possible effects of contact with the growth zone 2 series of Stage 1 sporangiophores were attached to the supporting slide in such a position that the apical growth zone was in contact with the petrolatum. The branching incidence was 10 to 20 % greater than in the controls, and the treated series which gave the larger value showed no internal damage in any specimen. There was no distinct effect on growth, and the average for treated sporangiophores (7.2 cm) was actually slightly greater than that of the controls (6.7). Banbury (1) found that lanolin-water-paraffin paste applied to the tips of Stage 1 sporangiophores was usually without obvious morphological effect.

The effects of water deficiency are illustrated in table V. Three series of Stage 1 sporangiophores were suspended from the slide with their bases 5 to 7 mm above the water level. The results show that the growth of otherwise undamaged sporangiophores was greatly reduced in every instance compared to the controls, and that branching increased in 2 of the 3 treated cultures. It is remarkable that these sporangiophores could grow unbranched as much as 4 cm, or produce branches, without an outside supply of

liquid water. A few sporangiophores even regenerated a slight amount of mycelium in air. One series of sporangiophores was tested without water in the dish, but there was no growth.

While water deficiency increases branching and decreases growth, the same effect can be obtained by immersion of the apical portions of Stage 1 sporangiophores in water. None of 20 individuals formed sporangia under these conditions, but in almost half there was a thickened region, which in some instances was so pronounced as to suggest an aborted sporangium. Although only few of these sporangiophores showed signs of damage due to handling, 65 % branched, sending out as many as 3, usually short, branches under water. A few branches grew vertically upwards into the air after 64 hours. The growth of immersed, unbranched, and otherwise undamaged specimens was exceedingly low (maximum 0.4 cm). A noteworthy feature of this type of growth is that 40 % of the sporangiophores regenerated hyphae from the base in air, and that a number of these hyphae were unusually long. The absence of sporangium formation, and the production of branches on immersed, isolated sporangiophores, is similar to Götze's (7) findings with Stage 1 sporangiophores on the mycelium immersed in water.

Negative Phototropism of Isolated Sporangiophores in the Ultraviolet: Grehn (8) reported that isolated sporangiophores remained sensitive to geotropic and phototropic stimuli, and Cohen and Delbrück (5) remarked that isolated sporangiophores growing on water are phototropic. Similar observations were made in the course of this work. Isolated sporangiophores gave positive phototropic curvatures in white light. In effect, the use of isolated sporangiophores for studies on phototropism represents one of the interesting applications of the technique.

Curry and Gruen (6) showed that normal Phycomyces sporangiophores gave strong negative curvatures in response to unilateral ultraviolet radiation. In order to find out whether isolated sporangiophores of the same strain respond in a similar way, they were grown on water, and exposed to unilateral ultraviolet (280 mμ, approximately 100 ergs/cm²/sec) between 20 and 30 hours after isolation. They responded in the same manner as the intact cultures giving strong negative curvatures of 90 to 150°. After rotating the curved isolated sporangiophores through 180° in the monochromator beam they curved back in the opposite direction. The development of curvature with time is probably quite similar to that in sporangiophores on the mycelium, since curvatures of 90° have been observed after 30 minutes.

Discussion

The technique described in this report permits the use of cultures of isolated upright sporangiophores for physiological studies. They can be used for long- or short-term growth measurements on any substance in solution, and can be grown in a row under controlled conditions. This arrangement represents a considerable advantage over the use of whole colonies even when grown only in small containers. A knowledge of the development and growth with time (fig 3) permits the use of isolated sporangiophores at a comparable stage of development, and at a predictable time after preparation of the cultures.

Isolated sporangiophores, after entering Stage 4, grow as well as those connected with the mycelium, but for a much shorter period. The final length is therefore less in isolated sporangiophores. It is deduced that sporangiophores of different length contain different amounts of major nutrients, at least in Stages 1 to 3, because of the sharp increase in dry weight during growth of attached specimens in Stage 1 (table III). One would expect then that sporangiophores of very different length would show differences in final growth if the deficiency caused by isolation involved only major nutrients. However, even the shortest sporangiophores (1 to 1.1 cm) isolated in Stage 1 grew only slightly less than longer ones, and differences in initial length between 1.2 and 4 cm did not affect the total growth at all (table II). Furthermore, sporangiophores isolated in Stage 2–3 probably have accumulated more materials than in Stage 1 because the dry weight in Stage 2–3 is generally slightly higher than in Stage 1 of the same length. But Stage 2–3 sporangiophores also failed to grow any better than individuals isolated in Stage 1. It seems more likely that the deficiency found in isolated sporangiophores (with the possible exception of the shortest ones) is of a subtler nature.

During Stage 4 there is some additional increase in weight in attached sporangiophores measuring 10 to 13 cm in length, but no increase from 5 to 10 cm. Apparently the dry weight reaches a plateau at about 10 cm length. The mycelium seems to continue supplying the sporangiophores with some materials in Stage 4, but the resulting increase in weight is disproportionately low relative to the pronounced total elongation during that stage, and compared to Stages 1 to 3. Part of the weight in Stage 4 is accounted for by the spore mass and by the extensive cell wall, and it is likely that growth in this stage involves primarily a conversion of cell contents to wall substance. This is also suggested by the data for isolated sporangiophores on water which form sporangia soon after isolation (fig 2) and then continue growing in Stage 4 to a considerable length even with a slight loss in dry weight. Although deposition of new cell wall also takes place in Stage 1 the rate of accumulation of cell contents accompanying growth must be quite high to judge from the increase in dry weight.

Two types of abnormalities were encountered in some isolated sporangiophores, internal damage and branching. The former is primarily due to excessive pressure during handling and is commonly accompanied by abnormally low growth in unbranched sporangiophores, but not necessarily by the production of branches except lateral branching from regions below the original apex. It may be that one of the contributing factors in the attendant decrease in

growth is a water deficit, since the response in sporangiophores deprived of liquid water is similar. Transport of water through the injured region is very likely impaired. However, when this region is immersed there is probably some uptake through the wall above. Grehn's agar block experiments also suggest water uptake through the wall. Another possible contributing factor to the decrease in growth of unbranched but injured sporangiophores is the partial loss to the growing apex of materials essential for growth, some of which are retained in the basal portion by blockage of transport at the injured region. The occasional production of lateral branches at, or close below, the damaged part supports this idea.

The latter phenomenon, lateral branching, is of interest from several points of view, especially because it represents an example of interference with apical dominance. Growth stoppage at the morphological apex (Stages 1 to 3) is usually associated with branching just below it, both in isolated sporangiophores, and, according to earlier investigators, in sporangiophores on the mycelium. It is a reasonable assumption that the growing apex normally prevents branching, possibly through a hormonal mechanism similar to that encountered in higher plants. When the apex stops growing under abnormal conditions, presumably through damage, the inhibitory mechanism is removed. The fact that elongation stops also under normal conditions during sporangium initiation and enlargement is no obstacle to the idea that apical dominance might be under hormonal regulation, since the young sporangium could still perform this function. But injury in the lower portions of a sporangiophore might make the apical dominance inoperative below the damaged region, and sporangiophore initiation could then take place if sufficient building materials are available. The idea that there is hormonal control of growth is only a working hypothesis, and the effect of injury deserves further study. Kirchheimer (11) found that sections of Phycomyces sporangiophores cut from the middle produced usually one branch solely at the apical end and below the cicatrization. However, treatment of the section with illuminating gas or camphor increased the branching and made it more basal.

In view of the abnormalities encountered in some of the isolated sporangiophores the question could be raised whether their failure to grow as well as normal ones (fig 3) might be merely because they are all injured to a greater or lesser degree during handling. However, it is only the final length of isolated sporangiophores which is less than that of individuals connected with the mycelium; the average growth rate is the same although it continues for a shorter time. In contrast with this, 7 measurements made on injured sporangiophores grown for 20 to 25 hours gave an average of 1.1 cm as compared to 3.5 cm for undamaged individuals. It is believed, therefore, that the slowing down of growth in isolated sporangiophores is due to the exhaustion of some material, not to any damage.

Summary

A technique is described which allows the culturing of isolated sporangiophores of Phycomyces on water or solutions. Sporangiophores isolated in Stage 1 underwent normal morphological development. After a period of little or no growth following removal from the mycelium, they initiated sporangia between 4 and 5 hours, and completed Stage 2–3 at about 13 hours after isolation (23° C and constant illumination). During Stage 4 they attained the same growth rate as that of sporangiophores attached to the mycelium. However, the isolated sporangiophores stopped growing between 60 and 80 hours, while comparable sporangiophores attached to the mycelium continued growing for 120 hours or more. The average final length of isolated sporangiophores was thus about 4 cm less than that of normal specimens. The growth of isolated sporangiophores after 80 hours was the same in light as in darkness, and the isolated sporangiophores did not significantly change the pH of the water during their growth.

The total growth of Stage 1 sporangiophores after 64 to 100 hours was independent of their initial length at the time of isolation for values between 1.2 and 4 cm. Those of 1.0 to 1.1 cm initial length grew on the average 0.4 to 0.6 cm less than the longer individuals. Sporangiophores isolated in Stage 2–3 grew less than those of any length isolated in Stage 1, and those isolated in Stage 4 grew less still.

In sporangiophores attached to the mycelium there was a pronounced increase in dry weight during Stage 1 which was almost directly proportional to the increase in length. In Stage 2–3 there was a slight further increase in weight, but no subsequent increase occurred until the sporangiophores, now in Stage 4, had attained a length of 10 cm, when an additional weight increase was observed. However, isolated sporangiophores grown for 64 to 67 hours on water suffered a slight loss in dry weight, and the final value was only about one half of that of sporangiophores of the same length which remained attached to the mycelium.

One sixth of the sporangiophores isolated in Stage 1, and grown for 40 to 100 hours, formed branches, an abnormal condition for Phycomyces. Of the 2 types of branching, apical and lateral, only the latter was usually accompanied by visible evidence of internal injury, the branch(es) arising from just below the damaged region. Often sporangiophores with such damage failed to branch, but the growth of most of these was abnormally low.

Both branching and reduction in growth could be induced experimentally by lack of liquid water or immersion of the growth zone in water, but excessive pressure applied near the base had no clear-cut effect on the branching incidence, although it increased the frequency of internal injury with the attendant decrease in growth.

Isolated sporangiophores give positive phototropic curvatures in white light, and strong negative curvatures when exposed to unilateral ultraviolet illumination (280 $m\mu$).

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GIBBERELLIN IN THE INDUCTION OF PARTHENOCARPY IN ZEPHYRANTHES [1, 3]

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Although fruit setting is known to be an auxin controlled phenomenon, not all plants can be made to produce parthenocarpic fruits by the artificial application of growth substances like IAA and other synthetic compounds (1, 2, 4). This has no doubt kept up the interest of the physiologist in discovering new chemicals concerned with fruit set.

Recently, Wittwer and his coworkers (8) have reported that gibberellins are remarkably efficient in producing parthenocarpy when applied to the floral parts of tomato. Preliminary trials with the unpollinated ovaries of cucumbers and egg plant have also yielded a similar response (6, 7). In the present investigation evidence is presented for the induction of parthenocarpy by gibberellin in a member of the Amaryllidaceae, Zephyranthes. In addition, the development of seeds lacking embryos within these parthenocarpic fruits is also reported.

[1] Received September 2, 1958.

[2] The bulbs of Zephyranthes were obtained from Calcutta Botanical Gardens.

[3] The plant has been tentatively identified as Zephyranthes x Lancasteri Traub by Dr. H. P. Traub, of La Jolla, California.

Methods

Zephyranthes[2], grown in the Varsity Botanic Garden, was selected for experimentation. The flower buds were emasculated and bagged 1 day preceding anthesis. The treatment was carried out the following day.

A hypodermic syringe was used to inject 0.5 ml of an aqueous solution of the chemical being tested into each ovary. Ten ovaries were used for each treatment. Since the ovary was unable to accommodate all of the treatment solution, a good portion of it was injected inside the hollow peduncle. Unpollinated controls and normally pollinated ovaries were also grown simultaneously.

A freshly prepared aqueous solution of gibberellin was employed. The gibberellin was obtained through the courtesy of Dr. L. G. Nickell, Chas. Pfizer & Co. Kinetin and IAA were dissolved in water with the aid of a few drops of HCl and NH_4OH respectively. Observations were recorded every 2 days until the fruits were completely ripe (2 weeks). The circumference of the fruit was used as an index of growth.

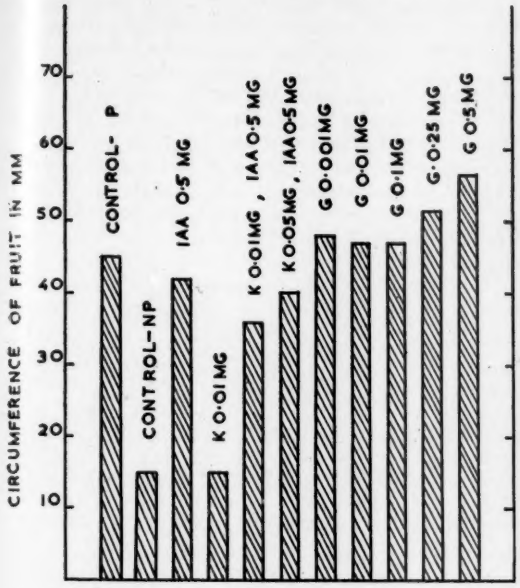

FIG. 1. Histograms showing the effect of various chemicals on fruit set. The slight increase of fruit size with G 0.001 mg over G 0.01, 0.1 mg is not considered significant. The pollinated and unpollinated controls have also been included for comparison. The unpollinated control ovaries represent the initial size since they do not show any growth. NP, nonpollinated; P, pollinated; K, kinetin; IAA, indoleacetic acid; G, gibberellin.

RESULTS AND DISCUSSION

The unpollinated controls shrivelled on the 4th day after emasculation (fig 1), thus establishing the absence of natural parthenocarpy. Unpollinated ovaries, treated with indoleacetic acid, produced parthenocarpic fruits, but these were smaller than naturally pollinated fruits and their shapes were also different (fig 2 A and B). The 3 locules appeared flaccid and the fruits had a more elongated outline. This was due to the absence of fully grown seeds within (fig 3 A and B). Only the fruit wall exhibited a positive response. The partition walls of the locules, instead of remaining thin as in controls, became swollen (fig 3 B). The behavior of the perianth tube also deserves mention. In pollinated ovaries it shrivelled and abscissed on the 4th day. In the treated ovaries it persisted throughout, turning dark green towards the base. There was also a prominent swelling at the junction of the pedicel and the peduncle and the latter became considerably swollen and curved. While the naturally maturing fruits dehisce 16 days after pollination, the IAA-treated fruits failed to open. Kinetin (10 and 50 ppm) had no stimulatory effect on the fruit wall or the seeds and both became attenuated as in the unpollinated controls.

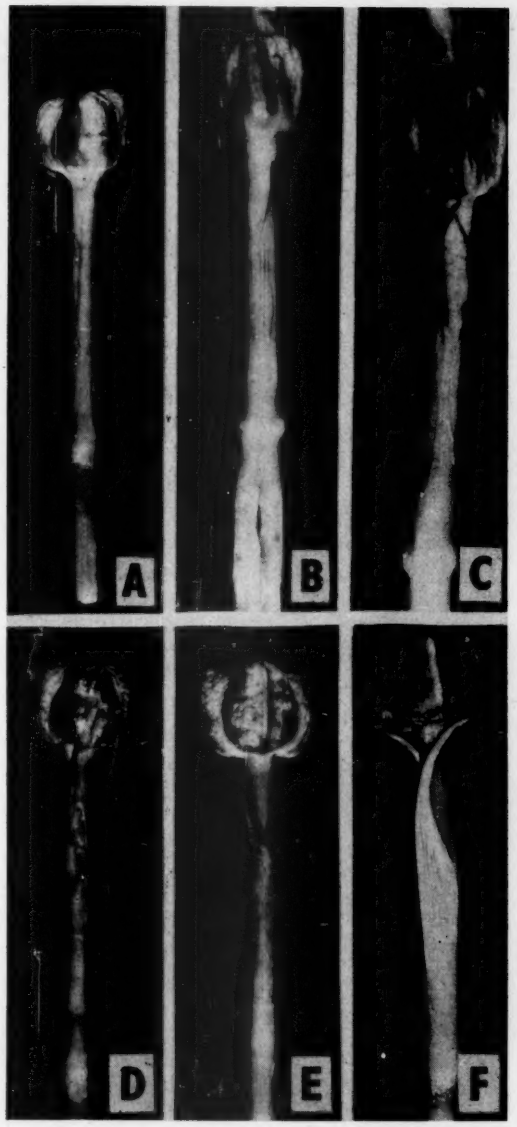

FIG. 2. Two-week-old normal and parthenocarpic fruits. A. Normal fruit from a pollinated ovary. B and C. Parthenocarpic fruits resulting from IAA (500 ppm) and IAA + kinetin (500 + 10 ppm) treatment respectively. D to F. Gibberellin-treated fruits; the concentrations employed were 10, 250 and 500 ppm respectively. × 0.11.

Mixtures of kinetin and IAA were tested to determine if they had any synergistic action. However, only seedless fruits with thickened walls appeared (figs 2 C, 3 A and B), indicating that kinetin was not active in fruit setting either alone or in the presence of IAA (fig 1).

Gibberellin induced fruit set over a wide range of concentrations (1 to 1000 ppm). In all the gibberellin-treated sets the fruit size was greater than that in the pollinated controls (fig 1). At concentrations lower than 500 ppm the size remained more or less constant, the largest fruits having been obtained with a concentration of 500 ppm (figs 1, 2 D to F). Higher concentrations (1000 ppm) gave no further increase in the size of the fruit.

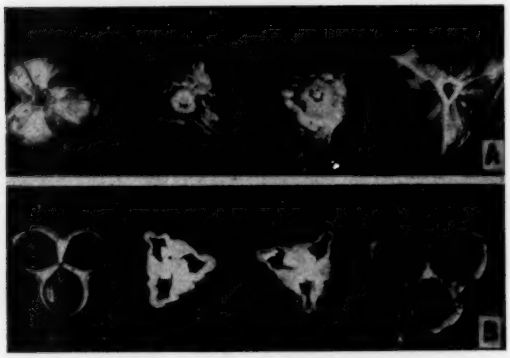

FIG. 3 A. Top view of mature fruits (2 weeks old). From left to right: naturally pollinated, treated with IAA (500 ppm), IAA + kinetin (500 + 10 ppm) and gibberellin (500 ppm). B. Same in cross section. The seeds in gibberellin-treated fruits are larger than pollinated controls. × 0.78.

The growth behavior during fruit development was similar to that in the pollinated controls (figs 3 A, B). There were no malformations of the vegetative parts and the treated fruits were practically indistinguishable from the normally pollinated ones except for their larger size. The gibberellin-treated fruits produced a greater number of seeds (fig 3 B) than the naturally pollinated ovaries. The smaller number of seeds in the naturally pollinated ovaries is due to a high degree of seed abortion (approximately 28 to 30 ovules out of 75 to 80 develop into seeds) whereas in the treated ovaries, almost all the ovules developed into seeds. The size of these seeds was also larger than those from naturally pollinated fruits (10 : 8 mm). However, dissections of seeds from the treated fruits showed no embryos.

Tomato is the only other plant extensively studied for its parthenocarpic response to gibberellin. However, it is not mentioned whether the parthenocarpic fruits were larger than or even as large as the naturally pollinated fruits (5, 8). Recently, Persson and Rappaport (3) induced fruit setting with gibberellin in a male-sterile tomato by spraying the foliage and also by soil application. This, according to them, indicated that, like auxin, gibberellin is also able to produce physiological effects remote from the site of treatment. Our observations are in conformity with the above findings, since most of the gibberellin was injected into the peduncle from where it presumably migrated upwards and brought about fruit setting. The ovaries of tomato, whether treated with auxin or gibberellin, produced only seedless fruits (7). This was not the case with Zephyranthes. Incidentally, it may be mentioned that so far gibberellins have not been reported to promote the growth of seed. Contrarily, there are a few reports saying that gibberellin can reduce the percentage of seed production (5).

It would be interesting to study the effect of gibberellins on plants in which auxin has failed to produce the desired results.

SUMMARY

The present study revealed that gibberellin is far more effective in bringing about fruit set in Zephyranthes than indoleacetic acid. It is of interest that this chemical is effective over a wide range of concentrations without producing abnormal effects on the vegetative and floral parts of the plant. In contrast, IAA resulted in the prevention of abscission of the perianth tube, while the peduncle became swollen and curved due to unequal growth.

Kinetin, used at 2 concentration levels (10 and 50 ppm) was ineffective in bringing about parthenocarpic fruit set. No synergism was observed between kinetin and indoleacetic acid. Gibberellin treated ovaries yielded fruits which exceeded even the naturally pollinated fruits in size. Although IAA-treated ovaries contained degenerating remains of ovules, with gibberellin the seed coat developed normally. The seeds lacked an embryo, but externally they looked like normal seeds. Seed setting was 98 to 100 %, while in nature it is only 40 to 42 %.

Grateful thanks are due to Professor P. Maheshwari for guidance and encouragement and to Dr. S. C. Maheshwari for some useful suggestions.

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METABOLISM OF CITRULLINE AND ORNITHINE IN MUNG BEAN MITOCHONDRIA[1]

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The occurrence of appreciable quantities of citrulline (31) and ornithine (26) in some higher plants and recently the discovery of trace amounts of these amino acids in others, such as barley and wheat (8) has led to speculations about their metabolism. Little information is available on the interconversions of these amino acids in plant tissue. It is known that when ornithine-2-C^{14} is administered to barley and white clover plants, the C^{14} label appears in citrulline, arginine, proline and glutamic acid (3). Further, Krebs and Eggleston (11) have detected citrulline phosphorylase activity in homogenates of the beans *Phaseolus lunatus* and *P. vulgaris*, and the pea, *Pisum sativum*. Beyond this, conjecture has had to rely mainly on data from work with bacteria and mammalian tissues.

It is possible that the mechanism by which citrulline is synthesized in plants is similar to that in extracts of *Streptococcus faecalis* (7) and rat liver (4, 7) and involves the following reactions:

$$ATP + CO_2 + \text{ammonia} = \text{carbamyl phosphate} + ADP$$

$$\text{carbamyl phosphate} + \text{ornithine} = \text{citrulline} + \text{inorganic phosphate}$$

The liver carbamyl phosphate synthetase was found to have a requirement for N-acetylglutamate which was unnecessary for *S. faecalis* extracts (14). Reichard (20) purified ornithine carbamyl transferase from liver and found it identical with the citrulline phosphorylase enzyme of Krebs et al (12).

Two feasible pathways that may exist in higher plants for the biosynthesis of ornithine are suggested by studies of the synthesis of ornithine from glutamate in the microorganisms *Neurospora crassa* and *Escherichia coli*. In *N. crassa*, ornithine arises from the transamination of glutamic-γ-semialdehyde (28), whereas in *E. coli* synthesis proceeds via several N-acetylated derivatives (27). It remains possible, of course, that yet another mechanism occurs in higher plants.

The present study is concerned with the enzymatic synthesis of citrulline and ornithine by mung bean (*Phaseolus aureus*) mitochondria and the role of carbamyl phosphate in the conversion of ornithine and citrulline.

MATERIALS AND METHODS

Mung bean seeds were soaked for 3 hours in tap water and then planted in moistened vermiculite contained in wooden seed boxes. The boxes were covered with glass plates to keep the humidity maximal. The seedlings were grown in the dark at 31° C for 2 days. Mitochondria were prepared from washed seedlings by the technique of Millerd et al (17) except that the preparative medium used throughout consisted of 0.1 M phosphate buffer (KH_2PO_4) pH 7.4, 0.4 M sucrose and 0.01 M disodium ethylenediamine tetracetic acid (EDTA). Mitochondrial suspensions were incubated with experimental media at 30° C.

Carbon dioxide was measured manometrically in the Warburg apparatus. Ammonia was determined by distillation in Conway units, followed by Nesslerization and measurement of the color produced in a Unicam spectrophotometer, Model SP 500, at 410 mμ. Citrulline was estimated by the modified ninhydrin reaction of Vogel and Bonner (29). Glutamic acid was determined manometrically using the glutamic acid decarboxylase of *Clostridium welchii* (10). Glutamic-γ-semialdehyde was assayed by the method of Vogel and Davis (30) using the mutant strains of *E. coli*, 55-25 and 55-1.

To isolate citrulline, ornithine and glutamic acid, reaction mixtures were deproteinized with sufficient glacial acetic acid to give a final concentration of 10 % acid, and then warmed gently for a few minutes. The protein was centrifuged off at 5,000 × G for 5 minutes. Aliquots of the deproteinized solution were banded onto paper strips (Whatman no. 3 MM), 5 cm × 40 cm. These strips were subjected to paper electrophoresis in a Shandon vertical electrophoresis apparatus using 0.1 M potassium bicarbonate as the electrolyte and a potential of 200 volts. A satisfactory separation of ornithine from citrulline and glutamate from aspartate was achieved in 2 hours under these conditions. Following drying at room temperature, the amino acids were located by spraying the edges of the bands with 0.05 M citric acid dissolved in acetone, then overspraying with a ninhydrin solution (0.1 % w/v in 95 % ethanol) and heating at 80° C for 10 minutes. The desired amino acids were each eluted from the paper with 5 ml of distilled water and the eluates evaporated to dryness. To free the amino acids from any radioactive contaminants, one dimensional paper chromatograms were run using diisopropyl ether : 90 % formic acid (3 : 2) (11) for ornithine and citrulline, and the organic layer of *n*-butanol : acetic acid : water (4 : 1 : 5) for glutamic acid. The solvent, 80 % aqueous phenol was also occasionally used for identification purposes.

The stimulatory effect of arsenate on citrulline

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phosphorylase (12) was used to measure the C^{14} content of the carbamyl group of citrulline. Citrulline phosphorylase was prepared from mung bean seedlings homogenized in sodium arsenate solution and sufficient hydrochloric acid to give a final concentration of 0.1 M arsenate buffer, pH 6.7. This homogenate was stored for 16 hours at 4° C to lower the endogenous carbon dioxide production and then used in the standard Warburg apparatus, under anaerobic conditions to decarboxylate citrulline. The activity of the homogenate was of the same order as that of mitochondrial preparations. Control flasks containing no citrulline and experimental flasks containing citrulline were used in duplicate with either alkali or water in the center well. The reaction was allowed to proceed for 3 hours, when 2 *N* hydrochloric acid was tipped into the reaction mixture to dispel the trapped carbon dioxide and stop the reaction. The $C^{14}O_2$ absorbed in 0.2 ml of *N* sodium hydroxide in the center well was estimated and from the total carbon dioxide produced, as measured manometrically, the specific activity of the carbamyl carbon was calculated.

Radioactive glutamic acid was degraded as described by Handler and Anfinsen (5). Carbon 1 was liberated as carbon dioxide by the action of *Cl. welchii* and the γ-aminobutyric acid produced was degraded by the Schmidt reaction. Trimethylene diamine dipicrate was isolated and the radioactive content measured.

Ornithine was decarboxylated using the acid permanganate reaction of Strassman and Weinhouse (23) and the resulting $C^{14}O_2$ was assayed for radioactivity.

All radioactive samples were assayed as described by Moses (18) using a windowless gas flow (methane) proportional chamber connected to a scaler.

N-acetylglutamate was prepared according to Nicolet (19). Carbamyl phosphate was prepared and stored as the lithium salt (7) and when required, converted into the barium salt (25). C^{14}-labeled compounds were obtained from the Radiochemical Centre, Amersham, England. O.P.C. 45, a non-ionic detergent was a gift from Petrochemicals Ltd. (London).

Results

Synthesis of Ornithine: To determine the pathway of the conversion of Krebs cycle intermediates to glutamate and ornithine, the incorporation of acetate-1-C^{14} into these amino acids was studied. Mitochondria in the presence of 1.3 micromoles (μM) of acetate-1-C^{14} (2×10^6 cpm) and 10 μM of malate and under the same experimental conditions as those in table I except that no amino acids were added, utilized 68 % of the initial acetate in 3 hours. Of this, 47 % was oxidized to carbon dioxide and 53 % was incorporated into mitochondrial substances. Malate was added to the experimental medium to supply ATP and oxalacetate (15) which are necessary for acetate oxidation (16). The oxidation of acetate-1-C^{14} indicates the presence of an acetate activating enzyme which has been previously demonstrated in crude extracts of mung bean seedlings by Millerd and Bonner (15). When mitochondria were incubated with acetate-1-C^{14}, malate and either glutamate or ornithine, 2 % of the acetate-1-C^{14} utilized was converted into glutamate and a smaller quantity into ornithine (table I). The low specific activity of ornithine, as compared with that of glutamate, indicates that ornithine is not so easily synthesized from α-ketoglutaric acid. The distribution of the C^{14} within the isolated amino acids is given in table I. Acetate-1-C^{14} was found to be incorporated into the carboxyl groups of glutamic acid, most of it being located in the γ-carboxyl group. This result implies that the tracer is incorporated by way of the Krebs cycle. Comparison of the C^{14} content of carbon 1 and of carbons 2 + 3 + 4 + 5 of glutamate and ornithine would seem to indicate derivation from a common carbon skeleton.

Studies were carried out on the conversion of glutamate-U-C^{14} (uniformly labelled) into ornithine by a system containing 0.1 μM of glutamate-U-C^{14} (100,000 cpm) and 20 μM of ornithine in the presence of a suspension of mitochondria. The mitochondria incorporated 8 % of the initial glutamate-U-C^{14} into ornithine. The addition of 0.001 M sodium arsenite

Table I

Distribution of C^{14} in Glutamate and Ornithine Following Incorporation of Acetate-1-C^{14} By Mung Bean Mitochondria

Amino Acid Added	Amino Acid at end of Experiment	Radioactivity incorporated	Specific activity	Percentage of total activity in carbon atoms			
	μM/flask	cpm	cpm/μM	1	2 + 3 + 4		5
Glutamate	44.2	28,370	642	9	0		88
Ornithine	48.1	3,120	65	13		87	

Complete system contained: 10 μM sodium 1-malate, 1.3 μM sodium acetate-1-C^{14} (2×10^6 cpm), 400 μM sucrose, 100 μM phosphate buffer pH 7.4, 10 μM EDTA, 20 μM $MgSO_4$, 1 μM ATP, 40 μg terramycin, 50 μM 1-amino acid and 1.0 ml of mitochondrial suspension. Total volume 2.0 ml. Temp, 30° C; gas phase, air; duration of experiment, 3 hours.

to the system, however, reduced the amount incorporated to 2%. Mitochondria oxidized ornithine to a limited extent (an uptake of 1.1 microatoms of oxygen per flask in 30 minutes). It was known from previous work that mung bean mitochondria contain traces of α-ketoglutaric acid (2) and, after the oxidation of ornithine, the system was found on analysis to have formed small amounts of glutamic-γ-semialdehyde and glutamic acid. Arsenite, at a concentration of 0.001 M, inhibited the conversion of glutamic acid to α-ketoglutaric acid. The inhibition by arsenite of the conversion of ornithine into glutamic acid and of glutamate into ornithine is of particular interest in view of Strecker's (23) finding that the synthesis of glutamic-γ-semialdehyde from glutamate by whole cells of a proline auxotroph of *E. coli* can be inhibited by arsenite.

The inhibition of aldehyde dehydrogenase by arsenite was studied by Jakoby (6), who found that the arsenite inhibition involved thiol groups. In view of this, it seemed a reasonable possibility that the sensitivity of ornithine oxidation to arsenite was due to the presence of thiol groups. The possible formation of a thioester of the γ-carboxyl group of glutamic acid was therefore investigated by using hydroxylamine as a trapping agent and estimating γ-glutamohydroxamic acid (13); no glutamohydroxamic acid was formed. Mitochondria did not phosphorylate glutamic acid. Glutamohydroxamic acid was only formed when glutamic acid, ATP, cysteine, magnesium sulphate, hydroxylamine were incubated with mitochondria. This is the condition for the assay of glutamine synthetase which has been shown to occur in bean mitochondria (32). Attempts to demonstrate the formation of glutamic-γ-semialdehyde from glutamic acid by mitochondria were unsuccessful. The conversion of ornithine to glutamic-γ-semialdehyde by the δ-ornithine-glutamate transaminase was demonstrated, however, in these mitochondria. It was found that 0.5 μM of glutamic-γ-semialdehyde was formed in 1 hour from 20 μM of ornithine and 20 μM of α-ketoglutarate at pH 7.4 by a heavy suspension of mitochondria. Glutamic acid, the other product of the reaction, was identified by one-dimensional paper chromatography. The presence of δ-ornithine-glutamate transaminase has recently been demonstrated in crude extracts of spinach leaves by Scher and Vogel (22).

INTERCONVERSION OF CITRULLINE AND ORNITHINE: Arsenolysis of citrulline which is inhibited by phosphate, is characteristic of the citrulline phosphorylase system (12). Mung bean mitochondria washed free from phosphate with 0.4 M sucrose solution were found to carry on a slow anaerobic decarboxylation of citrulline in the presence of 0.1 M sodium arsenate, pH 6.7, releasing 18 microliters of carbon dioxide per hour per flask. The presence of 0.05 M phosphate buffer, pH 6.7, inhibited the arsenolysis by 85%. Arsenolysis was found to produce 1 molecule of carbon dioxide and 1 molecule of ammonia from 1 molecule of citrulline. Ornithine was formed and identified by one-dimensional paper chromatography. Due to the low activity of the system, citrulline phosphorylase could not be directly demonstrated in these mitochondria by measuring the carbon dioxide released when citrulline was incubated with 0.1 M phosphate buffer.

However, 0.4 μM of citrulline were found to be synthesized in 1 hour from 20 μM of carbamyl phosphate and 20 μM of ornithine by mung bean mitochondria suspended in 0.05 phosphate buffer, pH 7.4. Under these conditions the phosphorolysis of citrulline is completely inhibited by the presence of ornithine. Citrulline was further identified by one-dimensional chromatography with 3 different solvents. This evidence suggest the presence of citrulline phosphorylase.

To study the synthesis of carbamyl phosphate by mitochondria, the following system was incubated for 1 hour at 30° C: 50 μM phosphate buffer pH 7.4, 10 μM ammonium chloride, 10 μM ATP, 10 μM N-acetylglutamate, 10 μM magnesium sulphate, 10 μM $NaHC^{14}O_3$ (200,000 cpm), 200 μM sucrose and 0.5 ml. of mitochondrial suspension to give a final volume of 1.0 ml. The control system contained no ammonium chloride. At the end of the experiment the reaction mixture was cooled to 0° C and the rest of the manipulations were carried out at 0° C. Twenty μM of lithium carbamyl phosphate were added to the reaction mixture and immediately followed by sufficient cold, concentrated perchloric acid to bring the pH of the system to 2.0. Carbamyl phosphate was isolated

TABLE II

DEGRADATION OF CARBAMYL PHOSPHATE BY MUNG BEAN MITOCHONDRIA

EXPERIMENT	ADDITIONS TO SYSTEM	CO_2 μL/HR/FLASK
1	None	6.1
2	$MgSO_4$	11.8
3	Glucose, ATP, Hexokinase	20.6
4	$MgSO_4$, Hexokinase, Glucose, ATP	29.8
5	$MgSO_4$, Hexokinase, Glucose, ATP, N-acetylglutamate	46.1
6	Expt 4, O.P.C. 45	63.2
7	Expt 5, O.P.C. 45	80.6

Each flask contained: 0.3 ml of mitochondrial suspension, 10 μM lithium carbamyl phosphate, 100 μM phosphate buffer pH 6.1, 400 μM sucrose and 10 μM EDTA. The additions to reaction were 10 μM $MgSO_4$, 50 μM glucose, 1 mg hexokinase, 4 μM ATP, 10 μM N-acetyl-1-glutamate and 0.1% (v/v) O.P.C. 45. Total volume 2.0 ml. Gas phase, N_2; Temp, 30° C. Additions were adjusted to pH 6.1. All results corrected for control system which contained boiled mitochondria, lithium carbamyl phosphate and $MgSO_4$.

from the deproteinized mixture as an ethanol-insoluble barium salt (25). This barium salt was dissolved in water, decomposed with normal hydrochloric acid in the Warburg apparatus and the liberated $C^{14}O_2$ was trapped and assayed. The barium salt obtained was tentatively identified as barium carbamyl phosphate by the method of isolation and by the rate of release of $C^{14}O_2$ compared with CO_2 produced from authentic barium carbamyl phosphate under acid conditions. It was possible under these conditions to obtain the synthesis of 0.1 μM of radioactive carbamyl phosphate.

The degradation of carbamyl phosphate by mung bean mitochondria could be more easily demonstrated than its synthesis (table II). Products of the reaction were ammonia and carbon dioxide. Increased decarboxylation at pH 6.1 was obtained with magnesium sulphate, the hexokinase system and N-acetylglutamate. Hexokinase and glucose convert ATP into ADP which reacts with carbamyl phosphate to form ATP, ammonia and carbon dioxide (4, 7). The inclusion of a non-ionic detergent, O.P.C. 45, which is an effective agent for disrupting the mitochondrial membrane (2), enhanced the degradation of carbamyl phosphate.

TABLE III

INCORPORATION OF $NaHC^{14}O_3$ INTO CITRULLINE BY MUNG BEAN MITOCHONDRIA

ADDITIONS TO REACTION SYSTEM	RADIOACTIVITY IN CITRULLINE (CPM)
None	5,800
N-acetylglutamate	6,080
N-acetylglutamate and Carbamyl phosphate	4,000
N-acetylglutamate with boiled Mitochondria	0

Each flask contained: 0.3 ml of mitochondrial suspension, 40 μM sodium succinate, 2 μM ATP, 10 μM $MgSO_4$, 20 μM l-ornithine, 20 μM NH_4Cl, 10 μM $NaHC^{14}O_3$ (200,000 cpm), 200 μM sucrose, 5 μM EDTA, 50 μM phosphate buffer pH 7.4. Additions to flasks, 5 μM N-acetyl-l-glutamate and 2 μM lithium carbamyl phosphate. Total volume 1.0 ml. Temp, 30° C; gas phase, air. At end of 1 hour, 10 μM of carrier l-citrulline added, immediately followed by deproteinization of the reaction systems.

That citrulline is synthesized by mitochondria at pH 7.4 from $NaHC^{14}O_3$, ammonium chloride, ornithine and a source of ATP is shown in table III. The presence of N-acetylglutamate had no effect on the synthesis of citrulline, but the isotopic dilution effect of added carbamyl phosphate indicates that carbamyl phosphate is an intermediate in the synthesis of citrulline. The citrulline isolated from this experiment showed 95% of C^{14} to be located in the carbamyl carbon.

DISCUSSION

It has been demonstrated by this series of experiments that acetate-1-C^{14} can be oxidized by mung bean mitochondria. If the assumption is made that this oxidation occurs via acetyl-CoA, which then enters the Krebs cycle, it would be expected that the labelling patterns produced in the intermediates of the cycle would be characteristic of acetate-1-C^{14}. After several turns of the cycle α-ketoglutarate would contain C^{14} in carbons 1 and 5 (21), and as these mitochondria contain glutamate dehydrogenase (2), the labeling in glutamic acid would also be located in carbons 1 and 5. The labeling pattern in glutamate which was in fact obtained, is consistent with the operation of a Krebs cycle in these mitochondria, if the presence of malate is taken into account. Malate acts as an isotopic diluent of malate-C^{14} so that the percentage of C^{14} in carbon 1 is decreased from the theoretical value of 33 % which would be achieved in its absence. The presence of a Krebs cycle was also indicated by the work of Millerd (15) on the oxidation of pyruvate by mung bean mitochondria. A similar isotopic pattern in glutamate was found by Bilinski and McConnell (1) when growing wheat plants were inoculated with acetate-1-C^{14}.

The presence of δ-ornithine-glutamate transaminase, the incorporation of glutamate-U-C^{14} into ornithine, and the inhibition of both this reaction and the oxidation of ornithine by arsenite, favors the synthesis of ornithine by the pathway occurring in *N. crassa* (28). That is, the synthesis of ornithine from glutamate in mung bean mitochondria involves the intermediate formation of glutamic-γ-semialdehyde, rather than, as occurs in *E. coli* (27), of acetylated derivatives of glutamate and ornithine. While the similarity of the distribution of C^{14} in glutamate and ornithine cannot be counted as direct support for the presence of this pathway, it is, however, compatible with its occurrence.

The synthesis of citrulline from ornithine, carbon dioxide and ammonia in mung bean mitochondria involves a carbamyl phosphate synthetase and citrulline phosphorylase, as was found to be the case with rat liver mitochondria and *S. faecalis* extracts (7). The behavior of N-acetylglutamate in mung bean mitochondria is different from that in either of these 2 systems since it activates carbamyl phosphate degradation while having no effect on citrulline synthesis.

SUMMARY

1. C^{14} from acetate-1-C^{14} is incorporated into carbons 1 and 5 of glutamate by mung bean mitochondria. The labeling pattern is consistent with the operation of the Krebs cycle.

2. Ornithine is synthesized from glutamate and ornithine-glutamate transaminase is present.

3. Citrulline is synthesized from carbon dioxide, ammonia and ornithine with carbamyl phosphate acting as the carbamyl group donor.

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News and Notes

Lewis Knudson
1884 – 1958

In his fifty-first year of scientific achievement following his initial appointment to the staff of the New York State College of Agriculture and Cornell University, Lewis Knudson, Emeritus Professor of Botany, died at his home in Ithaca on August 31, 1958. He is survived by his widow, 2 sons and several grandchildren.

Lewis Knudson was born in Milwaukee, Wisconsin, on October 15, 1884. Upon graduation from the University of Missouri in February, 1908, with the degree of B.S.A., he came to Cornell as assistant in plant physiology and began his teaching under Professor B. M. Duggar. He advanced to instructor at the end of the term, received the doctor's degree and was appointed Assistant Professor of Plant Physiology in 1911. Upon the resignation of Professor Duggar in 1912, he was made acting head of that department.

In 1916 the Department of Plant Physiology was incorporated into the newly created Department of Botany and Dr. Knudson became Professor of Botany.

During 1919–21 he spent a year in France and 6 months in Spain, lecturing in Madrid and Barcelona, carrying out research at the Sorbonne and attending lectures in the Pasteur Institute. In 1941, on the retirement of Professor Karl M. Wiegand, he became head of the Department of Botany. He retired, after 45 years with the College, on June 30, 1952.

Professor Knudson was an exceptionally effective teacher of both elementary and advanced courses in plant physiology and, during the absence of a colleague, also lectured with marked success in the general botany course. His sympathetic and stimulating direction of graduate work led 25 students to complete the work for the doctor's degree under his direction. Among these are some of the most widely known names in plant physiology, horticulture and related sciences.

In research, Dr. Knudson's work dealt with a variety of problems of fundamental importance to the field of plant physiology. His first major research was on tannic acid fermentation. Turning his interest then to a consideration of the physiology of the bacteria, he and his students developed a widely used method of culturing the nitrogen-fixing bacteria associated with legumes. His pioneering work on the organic nutrition of green plants produced highly useful methods of growing these plants in pure culture. This same work, applied to the non-symbiotic germination of orchid seeds, was to revolutionize the commercial growing of orchids. Professor Knudson also applied these pure culture techniques to the study of induced mutations in the haploid phase of ferns and demonstrated permanent changes in the chloroplasts by treating the fern spores with x-rays.

Professor Knudson's investigations of the physiology of the ripening of bananas, and on banana diseases, made major contributions to the economy of the Central American countries which raise this fruit. He also worked on rubber-producing plants in collaboration with several of his colleagues during and after World War II. Dr. Knudson's activities in consulting service and research continued after his retirement, and he had practically completed a monograph on the banana at the time of his death.

During his period of active service Dr. Knudson, in recognition of his clear judgment, was named to many of the most important committees of the Cornell faculty. Following retirement, he received the Gold Medal award of the Federated Garden Clubs of New York State in 1956 for "distinguished service in scientific research on the physiology and nutrition of plants," an honor accorded only one other scientist, the late Liberty Hyde Bailey. In 1957, Dr. Knudson was the recipient of an honorary Doctor of Science degree from his alma mater, the University of Missouri.

Dr. Knudson was a Fellow of the American Association for the Advancement of Science. His professional, scientific and honorary society affiliations included the Botanical Society of America, American Society of Naturalists, Real Sociedad Espanola de Historia Natural, American Society of Plant Physiologists, Sigma Xi, Phi Kappa Phi, Alpha Zeta and Gamma Alpha.

Dr. Knudson's distinguished contributions to science are in the record, and his inspiring lectures will live long in the memory of his students.

While he was famous as a research investigator and teacher, his innate human friendliness is also a memorial in the minds of many. However occupied he might have been with his own affairs, he was never too busy to listen sympathetically to the problems of others, and to offer kindly advice. He knew and loved many men of low and high degree and they in turn loved him. He enjoyed life to the full.—Daniel D. Clark